REMARKS

The specification was amended at page 5, second paragraph to clarify that the numbers were GenBank accession numbers, supported at page 8, last paragraph. Claims 1 and 7 are amended and claim 8 is cancelled herein. Claims 1, 3, 4, 6, 7, 9, 10, and 22-29, appear in the application for the Examiner's reconsideration. Claim 1 has been amended to state that "the one or more lactic bacteria that are not part of the resident microflora of the human mouth" as suggested by the Examiner. This amendment is supported, for example, on page 7, second full paragraph of the specification, defining "mouth" as the oral cavity of humans or animals. Claim 8 has been cancelled and its features incorporated into amended claim 7. As no new matter has been introduced, the entry of the claim amendments at this time is warranted.

Applicants appreciate the courtesies extended by Examiner Kathleen Kerr during an interview on September 4, 2003 with Applicant's attorney, Rodney J. Fuller. The comments appearing herein are substantially in accord with those presented and discussed during the interview.

The Examiner noted that a certified copy of the original foreign application must be filed with the U.S. Patent and Trademark Office in order to perfect the priority claim as the copy Applicants sent previously could not be found in Examiner's files. Applicants have ordered a second certified copy of the priority document and will submit the copy upon receipt.

The specification was objected to for having improper margins. Applicants submit herewith a substitute specification reprinted with proper margins and herein request that the substitute specification with proper margins be entered. No changes were made to he substitute specification other than to provide proper margins so that no new matter has been introduced. Applicants respectfully submit that the entry of the substitute specification should overcome this objection.

The Examiner further objects to the references "X17390," "X14490," and "X53657" as being unclear for not specifically stating that they are GenBank accession numbers. Applicants have amended the specification on page 5 to clarify that they are GenBank accession numbers. This amendment is fully supported by the specification, for example at page 8, wherein the references are expressly stated to be GenBank accession numbers.

The Examiner also objects to the term "FUM medium" as being indefinite. Applicants submit herewith, as requested by the Examiner, the references specifically demonstrate that the term "FUM" is understood by one skilled in the art to mean "Fluid

Universal Medium." *See* Gmür, R. and Guggenheim, B., (1983) Antigenic heterogeneity of Bacteroides intermedius as recognized by monoclonal antibodies, Infect. Immun. 42, 459-470; and

Loesche, WJ et al., (1972) The Predominant Cultivable Flora of Tooth Surface Plaque Removed from Institutionalized Subjects, Arch. Oral Biol. 17,1311-1325. Both articles are enclosed for the examiner's convenience.

Claims 1-3, 6-10 and 23-28 were rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter of the invention. Specifically, the Examiner states that the phrase "lactic bacteria that are not part of the resident microflora of the mouth" is unclear as to its exact metes and bounds. As agreed during the interview, Applicants have amended claim 1 to insert the term "human mouth" so that the phrase now reads, "lactic bacteria that is not part of the resident microflora of the human mouth." The art discloses extensive work on the microflora of the human mouth. In view of this amendment, one skilled in the art would understand the meaning of the above phrase. Therefore, Applicants respectfully request that this rejection be withdrawn.

Claims 2 and 6 were rejected under 35 U.S.C. 112, second paragraph, as being indefinite. Specifically, the Examiner objects to the term "about." Applicants have deleted the term "about" and request that this rejection be withdrawn.

Claim 3 was rejected under 35 U.S.C. 112, second paragraph, as being indefinite for the inclusion of the term "of dairy origin." Applicants respectfully traverse. The term "of dairy origin" is well known in the art. One skilled in the art would understand what the term "of dairy origin" means, as demonstrated by their use in the claims of other patent documents such as U.S. Patent Nos. 6,461,65 and 5,024,849 and by the article titled, "Detection, purification, and partial characterization of plantaricin C, a bacteriocin produced by a Lactobacillus plantarum strain **of dairy origin**" by Gonzalez, B., P. Arca, B. Mayo, and J. E. Suarez 1994 Appl. Environ. Microbiol. 60:2158-2163. Applicants respectfully request that this rejection be withdrawn.

Claims 1, 3, 4, 6-10, and 23-28 was rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to the skilled artisan that Applicants had possession of the invention when the application was filed. The Examiner states that to practice the claimed methods, the genus of low acidifying lactic bacteria is required as a reagent and, thus must be described. Applicants assert that the lactic bacteria has been described in the specification and that this rejection should be withdrawn.

First, the test for written description is whether the disclosure "reasonably conveys to the artisan that the inventor had possession" at the time of filing. Ralston Purina Co. v. Far-Mar-Co., Inc., 772 F2.d 1570, 1575 (Fed. Cir. 1985). *See also*, Amgen, Inc. v. Hoechst Marion Roussel, Inc., 314 F.3d 1313, 1331 (Fed. Cir. 2003). Satisfaction of this requirement, is measured by the understanding of the ordinarily skilled artisan. Lockwood v. Am. Airlines, Inc., 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997).

Applicants have submitted herewith a declaration by Dr. Rudolf Gmür as one skilled in the art. Dr. Gmür states that after reading the original specification, in view of the presently pending claims, that it is his opinion that Applicants were in possession of the presently claimed invention at the time of filing. Dr. Gmür bases his opinion on the fact that the original specification discloses the claimed method and provides exemplary compositions, five deposited lactic acid bacterial strains which have been deposited and further provides methods of making or isolating additional lactic acid bacterial strains that could be used in the presently claimed method.

Section 2163 of the MPEP explains that the examiner has the initial burden of presenting evidence of "why a person skilled in the art would not recognize that the written description of the invention provides support for the claims." The MPEP further emphasizes that "[t]here is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed."

Applicants point out that the claims are directed to a method of treating or preventing dental caries, dental plaque, and periodontal infection in a humans or animals by administering to the oral cavity one or more lactic bacteria that provide a pH in the oral cavity of 5.5 to 7 and that are capable of adhering directly to the pellicle of the teeth and not to an unknown DNA sequence.

The Examiner again relies on University of California v. Eli Lilly, 119 F.3d 1559 (Fed. Cir. 1997), to support her rejection stating that the concept of "requiring a precise definition, such as by structure, formula, or chemical name" is required to meet the written description requirement.

Applicants respectfully direct the Examiners attention to a more recent Federal Circuit case, Amgen, Inc. v. Hoechst Marion Roussel, Inc., 314 F.3d 1313, 1331-1333 (Fed. Cir. 2003), which explains Eli Lilly and helps clarify how the written description requirement is to be applied. Amgen related to recombinant technology related to the production of the hormone EPO, which controls the formation of red blood cells in bone marrow. The claims at dispute were the ones directed to non-naturally occurring EPO glycoprotein product and to

vertebrate cells and mammalian cells used in the production of EPO. Hoechst Marion Roussel argued that Amgen failed to sufficiently describe all vertebrate and mammalian cells as engineered in the claimed invention.

In providing its decision, the Court first pointed out that in Enzo Biochem, Inc. v. Gen-Probe, Inc., 296 F.3d 1316 (Fed. Cir. 2002) they had clarified that Eli Lilly "did not hold that all functional descriptions of genetic material necessarily fail as a matter of law to meet the written description requirement." In Enzo Biochem, the court held that a bacterial deposit was sufficient to meet the written description requirement, as one skilled in the art could obtain the claimed nucleotide sequences from the public depository and follow the appropriate techniques to excise the nucleotide sequences from the deposited organisms.

In Amgen, the Court stated that both Eli Lilly and Enzo Biochem were not applicable to the claims before them as the terms "vertebrate" and "mammalian" cells merely identified types of cells instead of undescribed, previously unknown DNA sequences. Instead the claims of Amgen's patent referred to types of cells that could be used to produce recombinant human EPO. The Court pointed out that, the words "vertebrate" and "mammalian" readily convey distinguishing information concerning their identity such that one of ordinary skill in the art could visualize or recognize the identity of the members of the genus.

Like in Amgen, Applicants are not attempting to claim some "undescribed, previously unknown DNA sequence." Also like Amgen where the terms "vertebrate" and "mammalian" conveyed a distinguishing description of the cells, the term "lactic acid bacteria" used in the presently pending claims also readily convey distinguishing information concerning their identity. As supported by the case law cited above, Applicants are not required to provide the DNA sequence of the lactic acid bacteria used in their method of treating or preventing dental caries in order to meet the written description requirement. But, even if under some new more stringent written description requirement such was required, Applicants would also meet such a requirement by the five lactic acid bacterial strains they deposited according to the Enzo Biochem decision explained above.

In view of the Federal Circuit decision in Amgen and as evident by the original specification, deposited lactic bacterial strains, methods of isolating other lactic acid bacterial strains that could be used in the method, and as supported Dr. Gmür's declaration as one skilled in the art, a skilled artisan would clearly understanding that Applicants were in possession of the presently claimed method of treating or preventing dental caries, dental plaque, and periodontal infection by orally administering low acidifying lactic bacteria

capable of adhering directly to the pellicle of the teeth. In view of the foregoing, Applicants respectfully request that the rejection for lack of written description be withdrawn.

Claim 7 was rejected under 35 U.S.C. 112, first paragraph, for lack of enablement. Applicants traverse. The Examiner states that while the specification is enabled for those methods using lactic bacteria modified to contain one of the three genes in Claim 8, Claim 7 is not enabled.

In an effort to expedite the prosecution of this application, claim 7 has been amended to incorporate the specific genes recited in claim 8. Furthermore, the specification teaches that the modification can be made using standard techniques know in the art and further teaches that it is preferably achieved according to the protocols described in Boumerdassi et al., Plattecuw et al., and Ito et al (page 9, first full paragraph). The specification also provides specific examples of how the genetic modification of the lactic bacteria can be achieved, *i.e.*, by insertion of the X17390, the X14490 or the X53657 gene, in order to improve adherence to the pellicle of the teeth or to be less acidifying than the resident microflora in the mouth (page 8, last paragraph). Since the specification provides both adequate guidance and specific examples of the modification, it is sufficiently enabling as undue experimentation would not be required to practice the claimed invention.

Claims 1-4, 6, and 9 are rejected under 35 U.S.C. 102(b) as being anticipated by WO 92/14475 by Madinier (hereafter "Madinier") and claims 1-4, 6 and 28 were rejected under 35 U.S.C. 102(b) as being anticipated by Busscher et al. (hereafter Busscher).

During the interview, the Examiner agreed that the prior art rejections would be moot if the term "about" was deleted from claim 1. Applicants have now amended claim 1 and respectfully request that the art rejections based on Madinier and Busscher be withdrawn, as they are now moot. Neither Madinier or Busscher teach or suggest a method of treating or preventing dental caries, dental plaque, and periodontal infection comprising administering to the oral cavity of a human or animal one or more lactic bacteria that are not part of the resident microflora of the human mouth, that are low acidifying, *i.e.*, having a pH of between 5.5 and 7, as presently required by the claimed invention.

In view of the above amendments and arguments, it is believed that the application is now in condition for allowance, early notification of such would be appreciated. Should the Examiner not agree, then a telephonic interview is respectfully requested to discuss any remaining issues and expedite the eventual allowance of the claims.

Respectfully submitted,

Date: Dec 9, 2003

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INCORPORATION OF EXOGENOUS LACTIC BACTERIA INTO ORAL MICROFLORA

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of the U.S. national phase designation of PCT application no. PCT/EP99/05473, filed July 26, 1999, the entire contents of which are incorporated herein by reference thereto.

FIELD OF THE INVENTION

The present invention relates to the incorporation of exogenous lactic bacteria into the oral microflora for the prophylaxis or the treatment of dental caries, dental plaque, and periodontal infection.

BACKGROUND OF THE INVENTION

The mouth (oral cavity) contains resident and non-resident microflora. The resident microflora includes microorganisms that are able to establish a more or less permanent residence on the oral surfaces. These bacteria are mainly localized on the tongue, the buccal mucosa, and the teeth while the gingiva, lips, checks, palate, and floor of the mouth only support a very sparse microflora.

On the tongue and the buccal mucosa, the natural resident microflora includes microorganisms selected from Streptococcus, Veillonella, Bacteroides, and Haemophilus. On the teeth, Streptococci, Lactobacilli and Actynomyces predominate but a variety of Gram positive and negative cocci and rods can be also found.

For example, Frandsen et al. showed that S. *sanguis* predominates on the buccal mucosa but its primary habitat is the surface of teeth, that S. *gordonji* grows in the mature supragingival plaque, and that S. *oralis* and S. *mitis* grow in the initial dental plaque (Oral Microbiol. Immunol., 6, 129-133, 1991). Strains belonging to the mutans group are localized on teeth (S. *criscetus, S. downei,* S. *ferus,* S. *macacae,* S. *mutans, S. rattus, S. sobrinus).* Strains belonging to the *S. milleri* group predominate in dental abscesses (S. *anginosus, S. constellatus, S. intermedius)* (Bentley et al., Int. J. System. Bacter. 1991, 41, 487-494; Wood et al., The Genera of Lactic Acid Bacteria, Blackie Academic and Professional, Chapman & Hall, W. H. eds., 1995).

Many of these microorganisms are innocuous commensal microorganisms, but a lot of them have been recognized as being the etiologic agent responsible for several

diseases (Hill, M. J. and Marsh, P. D. eds. Human Microbial Ecology, 1990, CRC Press, Boca Raton Florida, USA)

Dental plaque is a film that forms on the surface of teeth consisting of bacterial cells in a matrix of extracellular polysaccharide and salivary products. Immediately after eruption, the teeth are covered with an amorphous layer of saliva, the acquired enamel pellicle (AEP), that is about 1.3 μm thick and cannot be removed by normal tooth brushing. The deposition of bacteria on teeth immediately follows the formation of the AEP and plaque becomes evident in 8-12 hours as a multi-layered structure. The first layer consists of bacteria (earliest colonizers) that attach to teeth, mainly via specific adhesion-receptor recognition, and forms a substratum for the second colonizers that adhere one to the other by analogous specific binding or by simple juxtaposition. Plaque cohesion is essentially guaranteed by three mechanisms: the presence of a salivary pellicle on the outer bacteria layer, the specific co-aggregation among the different bacterial species, and the glucans synthesized by the bacteria that remain entrapped in the plaque matrix (Skopek et al., Oral Microbiol. Immunol., 2, 19-24, 1994; Kolenbrander et al., Meth. Enzymol., 253, 385-397, 1995; Hiroi et al., FEMS Microbiol Lett., 96, 193-198, 1992; Gibbons et al., Infect. Immun., 52, 555-561, 1986).

The organic acids produced by oral bacteria during the fermentation process directly cause dental caries. These acids attack the hard tissue of teeth with the consequent release of ions such as calcium, phosphate, carbonate, magnesium, fluoride, and sodium. When the pH in the oral cavity again increases to around neutrality, the saliva becomes saturated with calcium so that calcium liberation from the tooth is prevented. Among all the food residues found in the mouth, carbohydrates show the highest caries promoting effect since they are directly available for fermentation by oral bacteria.

Potentially all microorganisms that ferment sugars are cariogenic, but the primary etiological agents of coronal and root caries are the mutans streptococci because they are strong acid producers; Lactobacilli, that are highly aciduric, however, can also be implicated. In humans, *S. mutans* and S. *sobrinus* are the more cariogenic strains, and live on teeth while not colonizing the entire dentition. Their number is also less on anterior teeth than on molar teeth (Lindquist et al., Dent. Res., 69, 1160-1166, 1990). Moreover in human approximal plaque, *S. mutans* and *S. sobrinus* preferentially colonize the most caries-prone site apical to the contact area (Ahmady et al., Caries Res., 27, 135-139, 1993). A higher prevalence of S *sobrinus* was also found in the molar regions compared with that

of S. *mutans* (Lindquist et al., Caries Res., 25, 146-152, 1991).

S. mutans and *S. sobrinus* have been shown to attach to the pellicle of teeth mainly via specific adhesion-receptor interaction. Gibbons et al. showed that S *mutans* carries an adhesion which binds to salivary components in the pellicle, while *S. sobrinus* cells appear to possess an adhesion which binds to glucan in the pellicle (Infect. Immun., 52, 555-561, 1986).

The transient microflora comprise exogenous bacteria that are occasionally present in the mouth, but that do not establish a permanent residence therein (even if repeated oral administrations of these bacteria are carried out). All the food bacteria, and in particular lactic acid bacteria, can be part of this transient microflora. These exogenous lactic bacteria have never been shown to be capable of directly adhering to the pellicle of teeth. Repeated administration of exogenous lactic bacteria may, however, lead to colonization of the mouth on all the oral surfaces, such as the tongue, the buccal mucosa, the gingiva, lips, cheeks, palate, floor, and the teeth. This colonization may result from attachments via specific bindings to bacteria of the resident microflora (co-aggregation phenomena), via entrapment in the matrix of polysaccharide produced by the resident bacteria, or via adhesion to saliva proteins (especially glycoproteins).

Lactobacillus casei rhamnosus GG (ATCC53103) has been reported to colonize the mouth, most probably on the epithelium of the buccal mucosa. This strain also adheres to the epithelium of the intestinal tract (US Patent No. 5,032,399, Gorbach *et* al.; Micr. Ecol. In Health and Dis., 2, 295-298, 1994). By contrast *L. rhamnosus* does not adhere to teeth.

Japanese patent no. 4021633 (Cyconmedix KK) also reported colonization of the mouth by *Lactobacillus acidophilus*, most probably on the epithelium of the buccal mucosa. Many *Lactobacillus acidophilus* are known to also adhere to the epithelium of the intestinal tract (EP577904; EP199535; Perdigon et al., Medicina, 46, 751-754, 1986; Perdigon et al., Immunology, 63, 17-23, 1988).

Exogenous bacteria can also produce factors that inhibit the growth of the resident microflora in the mouth. For example, EP759469 (Société des Produits Nestlé) described the use of a bacteriocin produced by *Micrococcus varians* for inhibiting the development of the oral pathogens *S. sobrinus, S. sanguis, S. mutans,* and A. *viscosus*.

There are several strategies to minimize the development of resident microflora of the mouth. For example, by administering commensal bacteria of the resident

microflora that are not cariogenic, such as *Streptococcus salivarius* and/or Stomatococcus *mucilaginosus*, and/or repeated administration of exogenous lactic bacteria such as *L. casei*, *L. fermentum*, *L. acidophilus*, *L. crispatus*, *L. gasseri*, *L. salivarius*, *L bulgaricus*, and *S. salivarius* (Tanzer et al., Infec. and Immunity, 48,44-50, 1985; W092/14475).

The application of bacteriocins is another investigated strategy which has been used to reduce tooth caries. These molecules have attracted interest as prospective anti-carie agents and as factors important in modulating colonization of the oral cavity. The anti-carie potential of applying bacteriocins comes from their potent and broad antibacterial activity against mutans streptococci and bacteria associated with dental plaque and their natural occurrence in bacteria regarded as being safe to humans (US Patent No. 5,368,845 to Colgate, and WO 94/12150 to Smithkline Beecham).

The application of milk derivatives is also of interest for the health of the mouth. Indeed, US Patent No. 5,427,769 (Nestec S.A.) describes another alternative wherein dental caries are prevented by contacting teeth with an edible composition containing micellar casein in amount sufficient to inhibit colonization by *Streptococcus sobrinus*. EP748591 (Société des Produits Nestlé S.A.) also reports the use of fluoridated micellar casein or its micellar subunits for treating dental caries or plaque. US Patent No. 4,992,420 (Nestec S.A.) describes treatment of the buccal cavity with kappa-caseino-glycomacropeptide derived from milk for eradicating plaque and caries.

Lactic bacteria that are not part of the resident microflora of the mouth have never been shown to be really capable of directly adhering to the pellicle of teeth. By colonizing the surface of teeth, however, such lactic bacteria could exert an inhibitory activity against the growth of the resident microflora, including oral pathogens.

SUMMARY OF THE INVENTION

The present invention is directed to a method of treating or preventing dental caries, dental plaque, and periodontal infection in a humans or animals comprising administering to the oral cavity of a human or animal one or more lactic bacteria that are not part of the resident microflora of the mouth, that are low acidifying, and that are capable of adhering directly to the pellicle of the teeth to displace from the teeth or prevent attachment to the teeth of cariogenic strains of bacteria that are resident microflora of the mouth. In ne embodiment the lactic bacteria to be administered provides a pH in the oral cavity of about 5.5 to 5.7. Advantageously, the lactic bacteria may be of dairy origin.

The lactic bacteria is preferably one or more of *Streptococcus thermophilus*, *Lactococcus lactis subsp. lactis*, or *Lactococcus lactis subsp. lactis biovar diacetylactis*. In particular the lactic bacteria is one of the strains CNCM I-1984, CNCM I-1985, CNCM I-1986, CNCM I-1987, and LMG P-18997.

Preferably, the lactic bacteria has optimal growth at a temperature of about 37°C, *i.e.*, the temperature of the mouth. The lactic bacteria may have been genetically modified to have improved adherence to the pellicle of the teeth or to be less acidifying than resident microflora found in the mouth. The lactic bacteria may be genetically modified to have improved adherence to the pellicle of the teeth by insertion of the X17390 gene, the X14490 gene, or the X53657 gene.

In another embodiment the method of the invention further involves administering the lactic bacteria in combination with one or more of milk, fermented milk, milk derivatives, or bacteriocin. The milk derivative may be one or more of a caseino-glycomacropeptide, micellar casein, fluorinated micellar casein, or renneted milk.

The invention also relates to dental compositions for use in the methods of the invention. The lactic bacteria may be present in these compositions in an amount of 10^4 to 10^9 cfu/g in order to provide a pH of at least 5.5 when the composition is administered to the mouth of a human or animal. When bacteriocin is present in an the composition, it is typically present in an mount of 0.00001 to 50 percent by weight of the composition. When the milk derivative is one or more of a caseino-glycomacropeptide, micellar casein, fluorinated micellar caesin, or renneted milk it may be present in an amount of at least about 0.1 percent by weight of the composition. The composition may further include one or more of an oil soluble antioxidant in an amount of about 0.005 to 0.5 percent by weight of the composition and an abrasive. The composition may be in the form of a toothpaste, mouth rinse, gum, spray, beverage, candy, infant formula, ice cream, frozen dessert, sweet salad dressing, milk preparation, cheese, quark, yogurt, acidified milk, coffee cream, or whipped cream.

The invention also relates to a method for screening lactic bacteria capable of adhering to teeth. The method involves the steps of preparing monoclonal antibodies that recognize specific surface proteins of lactic bacteria strains that are capable of adhering to the teeth and screening lactic bacteria strains with the monoclonal antibody to identify the strains of lactic bacteria that adhere to teeth.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 represents the adhesion saturation curves for *S. sobrinus* OMZ 176 (1a), *L. lactis* NCC2211 (1b), and *S. thermophilus* NCC1561 (1c);

Figure 2 represents the effect of CGMP on the adhesion to S-HA beads of *S. sobrinus* OMZ 176, *L. lactis* NCC2211, and *S. thermophilus* NCC1561;

Figure 3 represents the effect of As-CGMP on the adhesion to S-HA beads of *S. sobrinus* OMZ 176, *L. lactis* NCC2211, and *S. thermophilus* NCC1561.

DETAILED DESCRIPTION OF THE INVENTION

The object of the present invention is to use lactic bacteria that are not part of the resident microflora of the mouth, that is lactic bacteria that are low acidifying and that are capable of adhering directly to the pellicle of the teeth, to prepare a composition intended for the prophylaxis or the treatment of dental caries, dental plaque, and periodontal infection.

In one embodiment of the invention the lactic bacteria have been genetically modified to increase its adherence to the pellicle of the teeth via adhesion factors and/or genetically modified to be even less acidifying, contributing to a pH in the oral cavity of about 5.5 to 7.

The lactic bacteria may be selected from the group consisting of:

- an acidifying lactic bacteria that adheres to the pellicle of the teeth and that has been genetically modified so that it is low acidifying compared to resident microflora;
- a non adherent lactic bacteria that is low acidifying and that has been genetically modified so that it adheres to the pellicle of the teeth;
- a non-adherent acidifying lactic bacteria that has been genetically modified so that it adheres to the pellicle of the teeth and genetically modified so that it is low acidifying compared to resident microflora.

In another embodiment the bacteria, that is not part of the resident microflora, is low acidifying compared to resident microflora and is capable of adhering directly to the pellicle of the teeth.

In another embodiment the composition for the health of the mouth comprises (1) at least a lactic bacteria that is not part of the resident microflora of the mouth, which is capable of adhering directly to the pellicle of the teeth and contributing to a

pH in the oral cavity of above 5.5, and (2) any form of caseinoglycomacropeptide, micellar casein, fluorinated micellar casein, renneted milk, or bacteriocin.

The invention also provides a method for screening lactic bacteria capable of adhering to tooth. The method comprises the steps of: (1) preparing monoclonal antibody recognizing specific surface proteins of a lactic bacteria strain capable of adhering to the teeth, and (2) screening any lactic bacteria strain by use of the monoclonal antibody of strain capable of adhering to the teeth.

The term "mouth," as used herein defines the oral cavity of humans or animals such as pets, composed by the oral mucosa (gums, lips, cheeks, palate, and floor of the mouth), the tongue, and the teeth (including artificial structures).

Resident microflora of the mouth includes all microorganisms that naturally live in the mouth because they can establish a permanent residence on the oral surfaces. The resident microflora of the mouth also includes bacteria that live in the interfacial region between the dental hard and soft tissues (the junction tooth-gingiva), even thought the gingival crevice and the periodontal pocket are not present in a healthy mouth. This microflora includes microorganisms selected from *Streptococcus, Staphylococcus, Enterococcus, Micrococcus, Peptostreptococcus, Peptococcus, Lactobacillus, Corynebacterium, Actinomyces, Arachnia, Rothia, Alcaligenes, Eubacterium, Propionibacterium, Bifidobacterium, Bacillus, Clostridium, Neisseria/Branhamella, Veillonella, Enterobacteriaceae, Campylobacter, Eikenella, Actinobacillus, Capnocytophga, Haemophilus, Simonsiella, Bacteroides, Fusobacterium, Porphyromonas, Prevotella, Leptotrichia, Wohlinella/Selenomonas, Mycoplasma, Candida, Spirochaetes,* Protozoa.

Transient microflora comprises exogenous bacteria that can be occasionally present in the mouth, but that do not establish a permanent residence. This transient microflora may comprise all the food micro-organisms, such as the bifidobacteria *(B. infantis, B. adolescentis, B. breve* and *B. longum);* the lactococci *(Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris,* and *Lactococcus lactis* subsp. *lactic biovar diacetylactis);* the streptococci *(Streptococcus thermophilus, S. lactis, S. lactis cremoris* and S. *lactis diacetylactis);* the Lactobacilli *(Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus helveticus, Lactobacillus farciminis, Lactobacillus alimentarius, Lactobacillus casei* subsp. *casei, Lactobacillus delbruckii* subsp. *lactis, Lactobacillus sake, Lactobacillus curvatus, Lactobacillus fermentum*; and the acidophile group comprising *L.*

johnsonii; (see Fujisawa et al., Int. J. Syst. Bact., 42, 487-491, 1992); the pediococci *(Pediococcus pentosaceus, Pediococcus acidilactici,* and *Pediococcus halophilus);* the enterococci; the staphilococci *(Staphylococcus xylosus* and *Staphylococcus carnosus);* the micrococci *(Micrococcus varians);* yeast of the genus *Debaromyces, Candida, Pichia, Torulopsis* and *Saccharomyces;* and mold of the genus *Aspergillus, Rhizopus, Mucor* and *Penicillium.*

The lactic bacteria according to the invention that are low acidifying and capable of adhering directly to the pellicle of the teeth that are used to prepare compositions for the prophylaxis or the treatment of dental caries, dental plaque, and periodontal infection displace pathogenic bacteria from the teeth or prevent the attachment of the pathogenic bacteria. The lactic bacteria according to the invention are "low acidifying," which means that they are less acidifying than pathogenic strains. Accordingly, they contribute to a pH in the oral cavity of about 5.5 to 7. Preferably, they are from dairy origin.

The lactic bacteria according to the invention adhere to the pellicle of the teeth via specific or unspecific interactions and/or adhesion factors. The specific adhesion factors are proteins or polysaccharides.

At least one lactic bacteria is selected from the group consisting of *Streptococcus thermophilus, Lactococcus lactis subsp. lactis,* and *Lactococcus lactis subsp. Lactis biovar diacetylactis* and particularly from the group consisting of the strains CNCM 1-1984, CNCM 1-1985, CNCM 1-1986, CNCM 1-1987, and LMG P-18997. These strains have been selected among lactic bacteria strains for their capacity to adhere to the pellicle of the teeth and their optimal growth temperature of about 37°C, which is the temperature in the oral cavity. Moreover they are capable of fermenting glucose and sucrose and do not synthesize glucans, which are factors leading to the pathogenicity of the cariogenic strains.

In one embodiment of the invention the lactic bacteria are genetically modifying so that they adhere to the pellicle of the teeth via adhesion factors. For lactic bacteria that already adhere to the pellicle of the teeth, this modification makes the strains more adherent to the surface of the teeth. In the same way, any non-adherent lactic acid bacteria (not Lactobacilli) can be genetically modified so that it adheres to the pellicle of the teeth. This modification of the lactic bacteria can be achieved, for example, by insertion of the genes X17390, X14490 or X53657 (GenBank accession numbers). These gene are responsible in S. *mutans* for the expression of the Antigen I/II that mediates adhesion to salivary glycoproteins.

According to the invention, it is also possible to genetically modify lactic bacteria so that they are low acidifying. For lactic bacteria that is already low acidifying this modification increases the effect by further decreasing lactic acid production. This modification can be achieved in many ways. Preferably, the modification is achieved according to one the protocols described in the following documents: Boumerdassi et al., Appl. Environ. Microbiol., 63, 2293-2299, 1997; Plattecuw et al., Appl. Environ. Microbiol, 61, 3967-3971, 1995; Ito et al., Biosci. Biotechnol. Biochem., 58, 1569-1573, 1994.

According to the invention, at least one lactic bacteria, genetically modified or not, is used in an "effective amount" for the preparation of compositions intended for the prophylaxis or the treatment of dental caries, dental plaque, and periodontal infection in humans or animals such as pets. This quantity is preferably between 10^4 to 10^9 cfu/g.

It is also possible to use the at least one lactic bacteria, in combination with milk derivatives, such as milk, fermented milk, or milk derivatives selected from any forms of caseino-glycomacropeptide, micellar casein, fluorinated micellar casein, renneted milk, or bacteriocin, for example.

Biochemical Characterization of the Selected Strains

Fermentation patterns: 49 simple sugars were tested with the api 50 CH bioMerieux strip test (bioM6rieux SA, 69280 Marcy-l'Etoile, France). The results are given in the Table 1.

Acidification curves: Acidification curves were determined at 37°C under the following conditions:

- *S. sobrinus* OMZ 176: FUM sucrose 1% and FUM glucose 1%
- *S. thermophilus* CNCM 1-1985: Belliker sucrose 1% and Belliker glucose 1%

Inoculation was always 5%. The pH was recorded every 20 min.

S..*thermophilus* CNCM 1-1985, from sucrose fermentation, lowers the pH to 4.5, while *S. sobrinus* OMZ 176 lowers the pH to 4.

Table I. Sugar fermentation of *L. lactis* CNCM I- 1987, *L. lactis* CNCM I-1986, *S. thermophilus* CNCM I-1984, S. *thermophilus* CNCM I-1985 and , *S. thermophilus* LMG P-18997.

Sugar	*L. lactis* CNCM I-1987	*L. lactis* CNCM I-1986	*S. th.* CNCM I-1984	*S. th.* CNCM I-1985	*S. th.* LMG P-18997
Adonitol	+++				
Aesculin	++	++++			
Amygdalin	++++				
D-Arabinose					
L-Arabinose					
D-Arabitol					
L-Arabitol	+++				
Arbutin	+++	+++			
Cellobiose	+++	+++			
Dulcitol					
Erythritol					
D-Fructose	+	++++			
D-Fucose					
L-Fucose					
Galactose	++	++++			
β-Gentiobiose		+++			
Gluconate					
2-keto-Gluconate					
5-keto-Gluconate					
GlcNAc	+	++++			
D-Glucose	+	++++	+	++	++
Glycerol					
Glycogen					
Inositol					
Inulin					
Lactose	+	++++	+++	++++	++++
D-Lyxose					
Maltose	++				
Mannitol	+++	++			

D-Mannose	+	++++			
Melezitose					
Melibiose					
α-Methyl-D-glucoside					
α-Methyl-D-mannoside					
D-Raffinose					
Rhamnose					
Ribose	++	++			
Salicin	+++	+++			
Sorbitol					
L-Sorbose					
Starch					
Sucrose			+++	++++	+++
D-Tagatose					
Trehalose	++				
D-Turanose	++				
Xylitol	+++				
D-Xylose					
L-Xylose					
β-methil-xyloside					

+, ++, +++, ++++ show if the fermentation begins after 3, 6, 24, or 48 hours, respectively.

The invention is also directed to compositions for the health of the mouth that comprise a lactic bacteria that is not part of the resident microflora of the mouth, that is low acidifying, and that is capable of adhering directly to the pellicle of the teeth. The compositions are particularly intended for the prophylaxis or the treatment of dental caries, dental plaque, and periodontal infection. The lactic bacteria strain may be selected from the group consisting of *Streptococcus thermophilus*, *Lactococcus lactis subsp. lactis*, and *Lactococcus lactis subsp. lactis biovar diacetylactis* and preferably from the group consisting of the strains CNCM I-1984, CNCM I-1985, LMG P-18997, CNCM I-1986, and CNCM I-1987. In these compositions the lactic bacteria strains may be genetically modified as described above.

The lactic bacteria strains may be included in a food, pet food, cosmetic, or

pharmaceutical composition, for example. Accordingly, the compositions are preferably a toothpaste, mouth rinse, gum, spray, beverage, candy, infant formula, ice cream, frozen dessert, sweet salad dressing, milk preparation, cheese, quark, yogurt, acidified milk, coffee cream, or whipped cream, for example.

In the compositions of the invention, the lactic bacteria strains may be included alone or in combination with milk derivatives, for example, in order to obtain synergistic preparations. Accordingly, these compositions for the health of the mouth comprise:

- a lactic bacteria that is not part of the resident microflora of the mouth, which is capable of adhering directly to the pellicle of the teeth;
- any forms of lactic glycopeptides, renneted milk, or bacteriocin.

The lactic glycopeptides are preferably caseino-glycomacropeptides (CGMP), fluorinated or non-fluorinated micellar casein (which can be obtained as described in EP 0 604 802 and EP 0 748 591), or renneted milk. The caseino-glycomacropeptides are preferably added in a minimum amount of about 0.1%. It has also been shown that the caseino-glycomacropeptides do not prevent the lactic bacteria from adhering to the teeth pellicle (Fig. 2 and 3).

Synergistic compositions may also be prepared by adding at least one bacteriocin which is active against Gram-positive oral bacteria. In this embodiment the oral hygiene compositions may comprise 0.00001 to 50%, and preferably from 0.00001 to 15% of purified bacteriocin, by weight of the composition. The bacteriocin is preferably variacin (EP 0759469).

To protect the composition from degradation, an oil-soluble antioxidant may also be included. Suitable antioxidants include the "tocopherols," butyl-hydroxyanisole (BHA), butyl-hydroxytoluene (BHT), and ascorbyl palmitate. The oil soluble antioxidant is present in amounts of from 0.005% to 0.5%, preferably 0.005% to 0.01% by weight of the composition.

Suitable abrasives for use in dentifrice compositions of the present invention include calcium carbonate, calcium aluminosilicate, alumina hydrates, alumina, zinc orthophosphate, plastic particles, and silica, of which silica is the preferred abrasive.

Compositions according to the invention will have a pH which is orally acceptable and within a range such that the activity of the lactic bacteria is not compromised. The pH may be in the range of 3.0 to 9.5, preferably in the range 3.5 to 6.5.

The compositions of the invention may be prepared by conventional processes that comprise admixing the ingredients together in the appropriate relative amounts and finally, if necessary, adjusting the pH to the desired value.

The invention is further directed to a method for screening lactic bacteria capable of adhering to tooth. This method comprises the steps of:

(1) preparing monoclonal antibodies that recognize specific surface proteins of a lactic bacteria strain capable of adhering to the teeth, and

(2) screening any lactic bacteria strain by using the monoclonal antibody of strain capable of adhering to the teeth.

The monoclonal antibodies are used as a tool to detect the said lactic bacteria strain among other strains growing nearby.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention, in addition to those described herein, will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the claims. Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties to the extent necessary for understanding the present invention. DNA manipulation, cloning and transformation of bacteria cells are, except where otherwise stated, carried out according to the textbook of Sambrook et al. (Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, U.S.A., 1989).

EXAMPLES

The examples are preceded by a brief description of the plasmids, strains, and the various media used, as well as the method for producing a monoclonal antibody.

The strains S. *thermophilus* S118 (NCC 1529), S123 (NCC 1561), *L. lactis subsp. Lactis* 29 (NCC 2211), *L. lactis subsp. lactis biovar dioacetylactis* 69 (NCC 2225) were deposited under the Budapest Treaty at the Collection Nationale de Culture de Microorganismes (CNCM 1-1984, CNCM 1-1985, CNCM 1-1986 and CNCM 1-1987, respectively), 25 rue du docteur Roux, 75724 Paris, France, on March 3rd, 1998. The strain *S. thermophilus* BF1 1116 (CNBL 1177) was deposited under the Budapest Treaty at the Belgian Coordinated Collections of Microorganisms LMG P-18997, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium, on July 5th, 1999. All restrictions as to the availability of these

deposits will be withdrawn upon first publication of this application or another application which claims benefit of priority to this application.

Example 1: Strains and Culture Conditions

More than 100 strains (belonging to the Nestlé culture collection) were screened for their ability to attach to saliva-coated hydroxyapatite beads, and in particular the following 23 strains: *S. thermophilus* Y54 (NCC 2284), *S. thermophilus* 5fi6 (NCC 1971), *S. thermophilus* Sfi13 (NCC 2008), *S. thermophilus* Sfi21 (NCC 2038), *S. thermophilus* Sfi39 (NCC 2130), *S. thermophilus* Sfi42 (NCC 2145), *S. thermophilus* Sfi47 (NCC 2172), *S. thermophilus* S118 (NCC 1529), *S. thermophilus* S119 (NCC 1536), *S. thermophilus* S122 (NCC 1554), *S. thermophilus* S123 (NCC 1561), *S. thermophilus* S126 (NCC *1587), L. lactis subsp. cremoris* 15 (NCC 92), *L. lactis subsp. cremoris* 25 (NCC 1932), *L. lactis subsp. cremoris* 136 (NCC 2419), *L. lactis subsp. diacetylactis* 8 (NCC 1970), *L. lactis subsp. diacetylactis* 28 (NCC 2057), *L. lactis subsp. diacetylactis* 69 (NCC 2225), *L. lactis subsp. diacetylactis* 80 (NCC 2272), *L. lactis subsp. lactis* 29 (NCC 2211), *L. lactis subsp. lactis* 50 (NCC 2224), *L. lactis subsp. lactis* 54 (NCC 2228), *S. macedonicus* 216 (NCC 2484).

The 5 oral strains, *S. sobrinus* OMZ 176, S. *oralis* OMZ 607, *A. naeslundii* OMZ 745, *V. dispar* OMZ 493 and *F. nucleatum* OMZ 596 were obtained from the Institute für Orale Mikrobiologie und Allgemeine Immunologie, University of Zürich and were cultured in FUM medium in anaerobiosis (GasPackSystem, BBL) at 37°C.

All the strains were stored in glycerol at -20°C and pre-cultured for 14 hours prior to use at their specific optimal temperature; *S. sobrinus* OMZ 176 grew in FUM medium lactococci and streptococci in M17 (Difco) except S. *thermophilus* NCC1529, S119, S122, NCC156l and S126 that grew in Belliker (prepared by dissolution of 20 g tryptone, 5 g yeast extract, 2.5 g gelatine, 5 g dextrose, 5 g sucrose, 5 g lactose, 4 g NaCl, 0.5 g Ascorbic acid, and 10 g beef extract in 1 L of water).

For plate counting, S. *sobrinus* OMZ 176 was cultured in Mitis-Salivarius agar (Difco), S. *thermophilus* NCC1529, S119, S122, NCC1561, BF11116, and S126 in Belliker agar (prepared by adding to liquid Belliker 15 g of Bacto agar, Difco), and the remaining lactic bacteria strains in M17 agar (Oxoid).

Example 2: Production of Monoclonal Antibody

A monoclonal antibody would be used as a tool to detect *L. lactis subsp. lactis* NCC2211 among 5 oral strains growing together on S-HA discs and forming a biofilm that simulates dental plaque. Therefore the monoclonal antibody was tested against these strains to verify there was no cross-reaction. To this end, the monoclonal antibody is produced as described by Granato et al. "A mouse monoclonal IgE antibody anti-bovine milk lactoglobulin allows studies of allergy in the gastrointestinal tract., Clin. Exp. Immunol., 63, 703-710, 1986.

Example 3: Selection of Adherent Lactic Bacteria

Attachment to saliva-coated hydroxyapatite beads (S-HA)

To select among the lactic bacteria dairy strains those strains that are able to attach to saliva-coated hydroxyapatite beads (S-HA), the procedure previously described by Neeser et al. (1994) was used with slight modification in that the bead washings were done with 150 μl volumes and Hyamine hydroxide was substituted with Benzethonium hydroxide (Sigma).

Briefly, all the strains were grown to the end of the log phase in FUM except *S. thermophilus* NCC1529, S119, S122, NCC1561, and S126 that were cultured in Belliker. S. *sobrinus* OMZ 176, *L. lactis subsp. lactis* NCC221 1, 50 and 54, *S. thermophilus* NCC1529, S119, S122, NCC1561, and S126 grew at 37°C, the remaining lactococci at 30°C, and the remaining streptococci at 42°C.

5 mg of hydroxyapatite beads (BDH Chemicals Ltd, Poole, England) were covered with 70 μl clarified saliva obtained from volunteers in the lab and prepared as previously explained (Neeser et al, 1994). Saliva coated beads were kept overnight at 4°C, then washed (first with distilled water and after with HEPES buffer) and finally inoculated with 100 μl of metabolically labeled bacterial suspension (bacteria had been grown in medium supplemented with 10 μCi/ml ^{14}C acetic acid). Adhesion took place during 45 min at 37°C, then unbound bacteria were washed away and the attached cells directly counted in a LKB scintillation counter (type 1219 Rackbeta).

Adhesion percentages are expressed as radioactivity bound to the beads relative to the total radioactivity added to each well. All measurements were done in triplicate. Table 2 reports the percentages of adhesion to saliva-coated hydroxyapatite beads obtained for several screened strains and for *S. sobrinus* OMZ 176 (the reference strain).

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Table 2: Percentages of Adhesion to Saliva-coated Hydroxyapatite Beads for Several Screened Strains.

STRAIN	% ADHESION (± SD)
***S. sobrinus* OMZ 176**	**2.23 ± 0.49**
S. thermophilus Sfi42 (NCC 2145)	0.08 ± 0.02
S. thermophilus Sfi47 (NCC 2172)	0.14 ± 0.04
S. thermophilus NCC1529	**2.89 ± 0.60**
S. thermophilus S119 (NCC 1536)	0.15 ± 0.04
S. thermophilus S122 (NCC 1554)	0.93 ± 0.17
***S. thermophilus* NCC1561**	**2.19 ± 0.50**
S. thermophilus S126 (NCC 1587)	1.19 ± 0.56
L. lactis subsp. diacetylactis 28 (NCC 2057)	1.59 ± 0.17
***L.lactis subsp. diacetylactis* NCC2225**	**1.96 ± 0.40**
L. lactis subsp. diacetylactis 80 (NCC 2272)	1.20 ± 0.35
***L lactis subsp. lactis* NCC2211**	**2.85 ± 0.85**

Four strains, *S. thermophilus* NCC 1529 (CNCM 1-1984), *S. thermophilus* NCC1561 (CNCM 1-1985), *L. lactis subsp. lactis* NCC2211 (CNCM 1-1986) (hereinafter *L. lactis* NCC2211) and *L. lactis subsp. diacetylactis* NCC2225 (CNCM 1-1987) showed adhesion values close to *S. sobrinus* OMZ 176.

L. lactis NCC2211 and *S. thermophilus* NCC1561 were chosen as the more promising candidates since they grow very well at 37°C, which is the temperature in the mouth, while *L. diacetylactis* NCC2225 has an optimal growth temperature of 30°C. In particular, *L. lactis* NCC2211 cannot grow on sucrose, but it can ferment a wide range of sugars, moreover other oral strain can provide glucose via their invertase.

Adhesion saturation curves

Curves of bound CFU versus CFU inoculated into the well were determined to verify if bead saturation could be obtained. The 50% saturation was obtained directly from the bending point of the curves. The adhesion saturation curves for *S. sobrinus* OMZ 176, *L. lactis* NCC2211, and *S. thermophilus* NCC 1561 were determined. They are shown in Figure 1.

For each of the three strains the CFU number inoculated in the well to get

50% bead saturation and the corresponding number of bound CFU were directly deduced from the bending point of the curves and are given in the table 3.

Table 3: Number of CPU Inoculated Per Well to get 50% Bead Saturation.

	cfu/well	Bound cfu	% adhesion
S. sobrinus OMZ 176	4.00 E+07	4.00 E+06	10%
L. lactis NCC2211	1.00 E+07	9.00 E-f-05	9%
S. thermophilus NCC156I	3.00 E+07	2.00 E+06	7%

Example 4: Effect of Caseino-glycomacropeptides

The influence of CGMP on the adhesion of *L. lactis* NCC2211 and *S. thermophilus* NCC1561 was studied to verify the possibility of using CGMP to foster the predominance of one of these two strains over pathogenic strains, namely *S. Sobrinus OMZ* 176. Caseino-glycopeptide (CGMP) and its desialylated derivative (As-CGMP) were obtained from Nestec S. A., Lausanne (for their preparation see Neeser et al., 1994).

The dose-response effect was studied on the adhesion to S-HA beads by inoculating, in the well, 100 µl of bacterial suspension (CFU/ml corresponding to the 50% bead saturation previously calculated) which contained CGMP or AsCGMP in different concentrations and then performing the adhesion assay in the usual manner. Concentrations in the range 0.05 to 3 mg/ml were tested. No previous incubation of the bacteria in presence of CGMP or As-CGMP was done.

Figure 2 provides the curves obtained for the three strains by plotting the number of bound cells versus increasing amounts of CGMP, the number of inoculated cells corresponds to 50% bead saturation formerly calculated for each strain. The strong inhibition observed in the case of S. *sobrinus* OMZ 176 confirms the previous results obtained by Neeser et al. (1994) and Schupbach et al. (J. Dent. Res., 75, 1779-1788, 1996).

Figure 2 shows that 0.25 mg/ml produced 50% inhibition of the adhesion of *S. sobrinus* OMZ 176, while more than 2 mg/ml were necessary to have the same effect with *S. thermophilus* NCC156I. CGMP slightly enhances the adhesion of L. *lactis* NCC2211.

As in the case of CGMP, the desyalilated derivative inhibits the adhesion of *S. sobrinus* OMZ 176; only 0.05 mg/ml are needed to produce 50% decrease in the adhesion percentage. As-CGMP does not influence *L. lactis* NCC2211 adhesion, while it slightly

fosters the adhesion of *S. thermophilus* NCC1561 (Fig. 3).

Example 5: Toothpaste

Toothpaste is prepared by adding 10^5 cfu/ml of at least one of the lactic bacteria strain CNCM 1-1984, CNCM 1-1985, CNCM 1-1986, CNCM 1-1987 or LMG P-18997 in a lyophilized form, to a mixture containing:

Cetyl pyridinum chloride	1.65%
Sorbitol (70% soln)	33.0%
Glycerin	25.0%
Sodium carboxymethyl cellulose	2.0%
Sodium fluoride	0.25%
Silica (RP 93)	26.3%
Thickening Silica (Sident 22)	8.1%
Sodium saccharine	0.5%
Poloxamer (Pluronic F 108)	3.2%

The toothpaste is intended for the prophylaxis or the treatment of dental caries, dental plaque, and periodontal infection.

Example 6: Ice Cream

A cream comprising 10.8% lactic fats, 13.5% milk solids (non fat), 0.3% Emulstab® SE30 and 0.3% Emulstab® foam (Grindsted, DK) is prepared and then pasteurized at 105°C for 20s, homogenized at 75°C and 300 bar, cooled to 38°C, and inoculated with pre-cultures in MRS medium, taken in the exponential growth phase, at a rate of 10^7 to 10^8 cfu/ml of at least one of the lactic bacteria strain of CNCM 1-1984, CNCM 1-1985, CNCM 1-1986, CNCM 1-1987 or LMG P-18997. The cream is then fermented for 10 hours at 38°C up to a pH of about 4.5. At the end of the fermentation, sucrose and glucose syrup is added thereto. The composition of the cream is presented in table 4 below. The mixture is then beaten, cooled to 4°C, stored at 4°C, and chilled to a degree of expansion of 95°C by volume.

Table 4: Ice Cream Composition

Ingredients	Composition (kg)	Fats (%)	Non-fat solids (%)	Sucrose (%)	Solids content (%)
Cream (35%)	30.83	10.79	1.54		12.33
Powdered skimmed milk	12.45		11.95		11.95
Emulstab® 5E30	0.41				0.37
Emulstab® foam	0.41				0.36
Water	55.91				
Total: cream base	100.00	10.79	13.49	-	25.01
Cream base	74.14	8.00	10.00	-	18.54
Sucrose	22.06			15.00	15.00
Glucose syrup	3.80				3.00
Fermented Ice cream	100.00	8.00	10.00	15.00	36.54

Example 7: Yogurt

5 L MRS culture medium were sterilized for 15 min at 121°C and then inoculated with 5% by volume of an active culture of at least one of the *S. Thermophilus* strains CNCM 1-1984, CNCM 1-1985, or LMG P-18997 containing approximately 10^9 cfu/ml. After incubation for 8 h at 41°C, a starter containing 4.5×10^8 cfu/ml was obtained.

5 L of reconstituted skimmed milk having a dry matter content of 10%, to which 0.1% yeast extract had been added, was sterilized for 15 min at 121°C and inoculated with 2% of an active culture of commercial thickening *Streptococcus thermophilus* containing approximately 10^9 cells/ml. After incubation for 4 h at 41°C, a starter containing 4.5×10^8 cells/ml was obtained.

One batch of whole milk containing 3.7% fats strengthened with 2.5% skimmed milk powder and then pasteurized for 30 min at 90°C was then inoculated with 2% by volume of the starter of at least one of the CNCM 1-1984, CNCM 1-1985 or LMG P-18997 strains and 3% by volume of the starter of thickening *Streptococcus thermophilus*. The inoculated milk is stirred, poured into pots, and incubated for 4 h at 41°C. The resulting yogurt obtained has a good firm and smooth texture and is intended for the health of the mouth.

Example 8: Chewing Gum

A chewing gum for preventing or treating dental caries, dental plaque, or periodontal infection can be prepared adding an active culture of at least one of the *S. Thermophilus* strains CNCM 1-1984, CNCM 1-1985, or LMG P-18997 so that it contains approximately 10^4 to 10^9 cfu/g, to the following typical ingredients:

Xylitol	67.5 %
Gum base	20%
Calcium carbonate	5 %
Glycerin	3 %
PluronicF127	2%
Cellulose gum	1 %
Ballast compounds	0.5%
Flavor	1 %

Example 9: Pet Food Composition

A pet food for mouth health is obtained by preparing a feed mixture made up of corn, corn gluten, chicken and fish meal, salts, vitamins, and minerals. The feed mixture is fed into a pre-conditioner and moistened. The moistened feed leaving the pre-conditioner is then fed into an extruder-cooker and gelatinised. The gelatinised matrix leaving the extruder is forced through a die and extruded. The extrudate is cut into pieces suitable for feeding to dogs, dried at about 110°C for about 20 minutes, and cooled to form pellets which have a water activity of about 0.6. The pellets are sprayed with 3 coating mixtures. Each coating mixture contains an active culture of at least one of the *S. Thermophilus* strains CNCM 1-1984, CNCM 1-1985, or LMG P-18997 but one coating mixture uses hydrogenated soy fat as a coating substrate, one coating mixture uses water as a coating substrate, and one coating mixture uses protein digest as a coating substrate. The pellets contain approximately 10^4 to 10^9 cfu/g of the strains.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: J. R. NEESER et al.

Application No.: 09/779,596

Filing Date: February 9, 2001

For: INCORPORATION OF EXOGENOUS LACTIC BACTERIA INTO THE ORAL MICROFLORA

Confirmation No: 6562

Group Art Unit: 1652

Examiner: Kathleen Kerr

Attorney Docket No.: 88265-406

DECLARATION OF DR. RUDOLF GMÜR UNDER 37 C.F.R. § 1.132

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Alexandria, VA 22313-1450

Sir:

I, Dr. Rudolf Gmür, do declare that:

1. I am a citizen of Switzerland and reside at Rehalpstrasse 31, 8008 Zürich, Switzerland.

2. I received a Ph.D. from the Institute for Cell biology of the Swiss Federal Institute of Technology in Zürich in 1977. From 1977-1979, I was an Associate Scientist at the Wistar Institute in Philadelphia and an Associate Scientist at the Institute for Immunology and Virology of the University of Zürich from 1979-1980. From 1981-1994 I was an "Oberassistent" at the Department of Oral Microbiology and General Immunology of the Dental Institute of the University of Zürich and "Lehrbeauftragter" of the University of Zürich. In 1988, I was awarded the H.R. Mühlemann Research Prize of the Swiss Society for Periodontology. From 1994 to present I have been "Wissenschaftlicher Abteilungsleiter" and "Privatdozent" or Professor (since 2001) at the Institute of Oral Microbiology and General Immunology of the Center for Dental, Oral Medicine and Maxiollofacial Surgery, at the University of Zürich.

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THE PREDOMINANT CULTIVABLE FLORA OF TOOTH SURFACE PLAQUE REMOVED FROM INSTITUTIONALIZED SUBJECTS

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Summary—Tooth surface plaque was removed from 11 institutionalized individuals and cultured on agar plates in an anaerobic chamber. The recovery of organisms on a dilute trypticase yeast extract medium (MM10), incubated anaerobically, averaged 33 ± 26 per cent of the microscopic count. Growth on MM10, under aerobic conditions averaged 8 ± 5 per cent. The anaerobe to aerobe recovery ratio on medium MM10 was about 4. Six hundred and seventy-one isolates grew on subculture and were partially characterized. About half the isolates were not capable of lowering the pH in glucose broth below 5·5. Streptococci accounted for about 38 per cent of the isolates and were found in each subject. A sub-group of 15 strains grew in 40 per cent bile, formed NH_3 from arginine, fermented salicin, but not inulin. These isolates possessed characteristics of *Streptococcus sanguis* and *Streptococcus mitis*. Various *Actinomyces* species comprised about 14 per cent and *Clostridium* species accounted for 8 per cent of the cultivable flora. *Bacteroides melaninogenicus*, *Fusobacterium* species, and *Veillonella* species each were about 6 per cent of the isolates. The overall character of these plaque isolates suggested that a gingival crevice microflora containing several amino-acid fermenting species had colonized the tooth surfaces. These organisms would not be expected to produce a plaque capable of decalcifying enamel, which might explain why these subjects had a low caries experience.

INTRODUCTION

THE DENTOGINGIVAL surfaces are colonized by a dense microbial population, i.e. $>10^{11}$ bacteria per gram wet weight (SOCRANSKY *et al.*, 1963; THEILADE and THEILADE, 1969; GORDON, STUTMAN and LOESCHE, 1971). This microbial flora is part of a complex ecosystem referred to clinically as dental plaque. Certain plaques, which form on the tooth and gingival surfaces, contribute to the disease processes which occur on or in these structures. As these pathologies have components which are dissimilar, i.e. decalcification leading to caries, and calcification leading to calculus formation, the bacteria isolated from these plaques should reflect these differences. Quantitative cultural studies have shown that tooth surface plaque obtained from individuals with low to moderate caries experience contains a high percentage of acidogenic organisms with *Streptococci* species and Gram-positive rods predominating (GIBBONS *et al.*, 1964). The preponderance of acidogenic organisms in tooth surface plaque is compatible with the decalcification that can occur on these surfaces. Gingival crevice plaque contains, in addition to the various acidogenic organisms, several species such as

3. I am a member of the International Association for Dental Research; American Society of Microbiology; and Schweizerische Gesellschaft für Zell- und Molekularbiologie. I am also presently a member of the Commission for the support of dental research of the Swiss Society of Dentists. I have worked as Referee for several scientific journals including the Journal of Microbiological Methods, the Journal of Periodontal Research, Medical Microbiology Letters, Microbiology, Oral Microbiology and Immunology, Anaerobe, Acta Medicinae Dentium Helvetica, and Systematic and Applied Microbiology. I have published over 55 papers in peer-reviewed journals and have written five book chapters or monographs.

4. I have read and understand the above-identified patent and the claims as presently pending, as well as the Office Action mailed July 10, 2003. I am making the following statements as one of ordinary skill in the art and in support of the patentability of the present claims.

5. The above-identified application is directed to a method of treating or preventing dental caries, or dental plaque. As presently claimed, the method comprises the step of administering to the oral cavity of a human or animal one or more lactic bacteria that are not part of the resident microflora of the human mouth, which lactic bacteria provide a pH in the oral cavity of 5.5 to 7 and are capable of adhering directly to the pellicle of the teeth, displacing or preventing cariogenic strains of bacteria from attaching to the pellicle of the teeth.

6. The specification describes a method of treating or preventing dental caries, or dental plaque by administering to the oral cavity one or more lactic bacteria that is low acidifying, providing a pH in the oral cavity of 5.5 to 7, not part of the resident microflora of the human mouth, and which is capable of adhering directly to the pellicle of the teeth, displacing or preventing resident cariogenic strains of bacteria from attaching to the pellicle of the teeth.

7. The original filed specification teaches and provides exemplary compositions that can be used with the claimed method to prevent or treat dental caries. The specification also discloses at least five different lactic bacterial strains that could be used in

the claimed method. Furthermore, the application teaches one skilled in the art how to isolate other strains that could alternatively used in the disclosed method if desired.

8. It is thus my opinion as one of ordinary skill in the art, after reviewing the presently pending claims in view of the originally filed application and its teachings, that Applicants were in possession of the presently claim invention (a method of treating or preventing dental caries, or dental plaque) at the time of filing.

9. I further declare that all statements made herein of my knowledge are true and all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this patent.

Dated: Nov. 21, 2003

Rudolf Gmür

Dr. Rudolf Gmür

Bacteriodes melaninogenicus, *Fusobacterium nucleatum*, *Treponema dentium*, and *Peptococcus* species (GIBBONS *et al.*, 1963) which derive energy from amino acid catabolism with the formation of ammonia and various fatty acids (LOESCHE, 1968). Ammonia as a strong base would be available to neutralize the acids formed from fermentation and to act as a tissue irritant (MACDONALD and GIBBONS, 1962; RIZZO, 1967). The presence of these amino-acid fermenting species might explain why gingival plaque pH is close to neutrality (KLEINBERG, 1970) and consequently why calculus formation and not caries is a likely event in the gingival crevicular area.

The present investigation of tooth surface plaque in institutionalized subjects was undertaken in order to determine whether their plaque flora would reflect the clinic differences between them and the population sampled by GIBBONS *et al.* (1964).

MATERIALS AND METHODS

Subjects

Eleven institutionalized subjects, aged 11–22 yr and residents of a single dormitory at the Plymouth State Home and Training School, Northville, Michigan, were studied. At the time of sampling, these subjects had a gingivitis score of $2 \cdot 54 \pm 0 \cdot 44$ (RAMFJORD, 1959). About 19 ± 23 mg plaque dry weight could be removed from the buccal surfaces of the 6 teeth used in the Ramfjord scoring system. The higher plaque weights were due to heavy calculus deposits in some individuals. No mongoloids were included. The subjects had various neurological and mental disorders and some were under medication for these problems.

Sample collection

The oral flora appears to be sensitive to short exposures to atmospheric oxygen (GORDON, STUTMAN and LOESCHE, 1971). Because our samples were collected in the field and were some 2–3 hr away from the anaerobic chamber, precautions were taken to minimize oxygen exposure of the samples. The teeth to be sampled were not air dried. Buccal surface plaque was removed with periodontal scalers from the maxillary left second premolar, maxillary left first molar, mandibular right second premolar, and mandibular right first molar. The plaque was immediately placed in 1 ml of a reduced transport fluid (RTF), Table 1, contained in a shell vial. The shell vials containing the samples were cork stoppered and had minimal, if any, air space. The vials were placed into a jar made anaerobic with a Gas Pak (Bioquest) and returned to the laboratory. The RTF contained no nutritives and because of the dithiothreitol (DTT) would be expected to have a low Eh (CLELAND, 1964). Plaque from other teeth in the subject was removed at the same time, weighed, and its microscopic bacterial count determined. As the microscopic counts of the cultured plaque were also determined, it was possible by a simple proportional equation to calculate the weight and bacterial density of the cultured plaque.

Sample dispersion and plating

The vial containing the plaque was introduced into a plastic anaerobic chamber (ARANKI *et al.*, 1969) containing an atmosphere of 85 per cent N_2, 10 per cent H_2 and 5 per cent CO_2. All subsequent manipulations and incubations were performed in the chamber. The aluminium foil around the vial and the cork stopper were removed from the vial. The vial was dropped intact into 100 ml of RTF contained in a Waring Blender. The vial and its contents were then disrupted by mixing in the blender for 2 min. Aliquots from the blender dilution were: (1) stained with buffered formalin gentian violet dye and a microscopic count was obtained using the Petroff Hauser counting chamber (ARANKI *et al.*, 1969; SPEARS and FRETER, 1967); (2) saved for subsequent fluorescent antibody examination; and (3) carried through a serial 10-fold dilution in RTF. The buffered formalin gentian violet dye had been passed through a $0 \cdot 45$ μm membrane filter to remove particles which could be confused with bacteria. The aliquot from the blender dilution was mixed by a Vortex mixer prior to counting. Each suspension was stained with the gentian violet and allowed to settle for 30 min in the counting chamber. Duplicate counts were made routinely; and when the results differed by more than 10 per cent, repeat determinations were performed. All counts were performed as soon as possible after collection by the same individual.

TABLE 1. COMPOSITION OF REDUCED TRANSPORT FLUID, MODIFIED M10 MEDIUM AND CHARACTERIZATION BROTH

Reduced transport fluid (RTF)	Additions per 100 ml
0·04% Na_2CO_3*	0·5 ml of stock solution 8% Na_2CO_3
0·001 M EDTA	1·0 ml of 0·1 M EDTA stock solution
0·02% Dithiothreitol*	2·0 ml of 1% DTT stock solution
0·045% $K_2H\ PO_4$	7·5 ml of 0·6% K_2HPO_4 stock solution
0·045% $K\ H_2PO_4$	7·5 ml of stock mineral solution containing
0·09% NaCl	0·6% KH_2PO_4 (SMS)
0·09% $(NH_4)_2SO_4$	1·2% NaCl (SMS)
0·018% $MgSO_4$	1·2% $(NH_4)_2SO_4$ (SMS)
	0·25% Mg SO_4 (SMS)
MODIFIED M10 MEDIUM†	
1·5% agar	
0·2% trypticase	
0·05% yeast extract	
0·024% K_2HPO_4	3·8 ml of 0·6% stock solution
0·024% KH_2PO_4	
0·045% NaCl	
0·045% $(NH_4)_2SO_4$	3·8 ml of stock mineral solution
0·009% $MgSO_4$	
0·1 mg% haemin	1 ml of 10 mg% stock solution
0·025% KNO_3	0·5 ml of 5% stock
Autoclave above, then add aseptically	
3% sheep blood	
0·1% glucose	1 ml of stock 10% glucose
0·04% Na_2CO_3*	0·5 ml of 8% Na_2CO_3
0·012% cysteine*	0·5 ml of stock 2·5% solution
0·01% dithiothreitol*	1 ml of stock 1% DTT
Menadione* 0·5 μg/ml	0·1 ml of stock containing 50 mg/100 ml
Fatty acids	0·5 ml of stock solution containing
	Acetic 17 ml
	Propionic 6 ml
	N-butyric 4 ml
	Iso-butyric 1 ml
	N-valeric 1 ml
	Iso-valeric 1 ml
	DL-2-methyl-*n*-butyric 1 ml
	Lactic (88%) 5 ml
	Formic (88%) 2 ml
	Make up to 100 ml with sterile distilled H_2O
CHARACTERIZATION BROTH	
1% tryptone	
0·5% yeast extract	
1% veal infusion broth	
5% gelatin	
0·025% KNO_3	0·5 ml of 5% stock
0·1 mg% Haemin	1 ml of 10 mg% stock
0·024% K_2HPO_4	3·8 ml of 0·6% stock
0·024% KH_2PO_4	
0·045% NaCl	
0·045% $(NH_4)_2SO_4$	3·8 ml of stock mineral solution
0·009% $MgSO_4$	
Autoclave above, then add aseptically	
0·5% glucose	
0·12% cysteine	
Menadione 0·5 μg/ml	0·1 ml 50 mg% stock

* Filter sterilized 0·22 μm filter.
† Enough sterile 40 per cent NaOH is added to pH the medium to 6·8–7·2.

After the plaque suspension had been serially diluted, suitable aliquots were placed on mitis-salivarius tellurite agar medium (MST) (Difco) and on a supplemented dilute trypticase, yeast extract agar, Table 1. This medium, designated hereon as MM10, is a modification of the M10 rumen fluid formulation of CALDWELL and BRYANT (1966), and was an attempt to provide the known nutrient requirements of the oral flora (LOESCHE and GIBBONS, 1966) in the hope that these additives would permit the growth of more plaque species. Because of the complexity of the medium, it was prepared

in large batches and frozen until used. Subsequent studies suggested that the volatile fatty acids could be omitted (unpublished data).

A membrane filter technique was used to harvest bacteria from the serial dilutions. One ml aliquots of the appropriate dilution were placed on the surfaces of 0·22 μm filters supported in a plastic filtering apparatus (Sterifil Unit, Millipore Corp., Bedford, Mass.). The liquid was drawn through the filter and then the filter containing the impinged bacterial cells was placed on the surfaces of the agar media. This filter procedure gave slightly higher counts when compared with the glass rod spreading technique. As the filters could be removed and saved, this procedure provided a means of preserving the colonies that were present in the primary isolation. Conceivably, at some future time, upon development of suitable fluorescent antibody reagents, these colonies could be identified or confirmed (DANIELSSON, 1965; JABLON, FERRER and ZINNER, 1971).

Partial characterization of isolates

The MST plates were incubated anaerobically for 1–2 days and then left overnight aerobically at room temperature. Colonial growth on this medium was examined under the dissecting microscope, and colonies resembling *Streptococcus mutans*, *Strep. sanguis*, and *Strep. salivarius* enumerated. The MM10 plates were incubated both anaerobically and aerobically. After 5–7 days incubation, the number of colonies on the plates were counted. One anaerobic MM10 plate from each child containing well isolated colonies was selected for further study. Each colony was subcultured on MM10 agar for a purity check. This was necessary because some of the primary colonies were mixtures of 2 or more bacterial types on Gram stain. Isolates pure by Gram stain and darkfield examination were subcultured to a characterization broth (CB), Table 1. After growth in this medium, the following tests were performed: terminal pH, H_2S production determined by blackening of lead acetate strips, nitrate reduction, gelatin liquefaction, and indole production following methods described by the SOCIETY OF AMERICAN MICROBIOLOGISTS (1957). Growth from the CB was streaked on MM10 plates and incubated aerobically. Gram stain and darkfield examinations of growth in the CB were performed. Representative strains were subcultured in peptone yeast extract glucose broth (PYG). After growth occurred, the acid end-products were determined using the gas-liquid chromatographic procedures recommended by the Anaerobic Laboratory at Virginia Polytechnic Institute (ANAEROBE LAB., 1970).

RESULTS

A major difficulty in the quantitative recovery of bacteria from a dense microbial mat such as plaque is the dispersion and separation of the aggregation into single cells. The plaque obtained from these institutionalized subjects was difficult to disperse into single cells as judged by microscopic observation. Each cell or clump was counted as a single microscopic unit and the recoveries on the various media are reported as a percentage of the total number of microscopic units. A significant improvement in microscopic unit count was achieved by the addition of 0·001 M EDTA to the RTF. The original formulation for the RTF had 0·0045 per cent $CaCl_2$ and no EDTA. Plaque collected and dispersed in RTF with $CaCl_2$ had a microscopic density of $6 \cdot 6 \pm 4 \cdot 5 \times 10^8$ cells/mg dry weight. When the $CaCl_2$ was deleted and 0·001 M EDTA added to the RTF, the microbial density increased two-fold to $13 \cdot 0 \pm 4 \cdot 3 \times 10^8$/mg dry wt. This difference was highly significant by the Student *t*-test, i.e. $p < 0 \cdot 01$, where $n = 74$. Even so, about 10–15 per cent of the microscopic units in the EDTA treated plaque contained 2 or more cells.

The dispersed plaque dilutions were plated 2–3 hr after their collection. The recovery of organisms on anaerobically incubated MM10 medium averaged $33 \cdot 3 \pm 25 \cdot 9$ per cent (Table 2) of the microscopic count. Growth on MM10 under aerobic conditions averaged $8 \cdot 3 \pm 5 \cdot 4$ per cent, whereas growth anaerobically on the MST medium averaged $7 \cdot 6 \pm 9 \cdot 0$ per cent. The anaerobic to aerobic recovery ratio on MM10 medium was about 4 (Table 2).

TABLE 2. RECOVERY OF BACTERIA FROM SUPRAGINGIVAL PLAQUE OF INSTITUTIONALIZED CHILDREN (n = 11)

Media	Viable count	% Recovery*	
MM10†			
Anaerobic	4·3 ± 3·3 × 10⁸§/mg dry wt	33·3 ± 25·9§	p = 0·01
Aerobic	1·1 ± 0·7	8·3 ± 5·4	
MST‡	1·0 ± 1·2	7·6 ± 9·0	

* Recovery = viable count divided by microscopic count × 100.
† Modified M10 medium: CALDWELL and BRYANT (1966).
‡ Mitis salivarius tellurite medium.
§ Plus or minus standard deviation.

Six hundred and seventy-one isolates grew on subculture and were partially characterized. A considerable number of these isolates were not capable of lowering the pH in the characterization broth below 5·5 (Table 3). Thirty-three per cent of the isolates

TABLE 3. PREDOMINANT CULTIVABLE FLORA OF INSTITUTIONALIZED CHILDREN

Subject	Number of isolates	Per cent distribution of isolates according to terminal pH in glucose broth (CB)*		
		< 5·5 pH	5·5–6·5 pH	> 6·5 pH
J.L.	66	67	1·5	31·5
E.G.	47	70	8·5	21·5
C.M.	67	65·5	3	31·5
L.H.	64	45	19	36
C.C.	76	38	12	50
D.B.	46	46	26	28
B.B.	85	48	18	34
J.B.	76	44	9	47
W.J.	40	32·5	33	37·5
B.G.	37	54	24	22
R.S.	67	49	28·4	23
Total	671	50·8 ± 12·1†	16·6 ± 10·5†	32·9 ± 9·4†

* CB characterization broth.
† Plus or minus standard deviation.

appeared to be nonfermentative and 17 per cent were weakly fermentative as they lowered the pH between 5·5–6·5. The isolates were grouped according to the limited number of tests performed. The major types of bacteria found could be placed in recognized genera and given species identification in some instances. The response of the isolates in the NO_3 and H_2S tests facilitated the grouping of the unidentified organisms. A summary table showing the main groupings is given in Table 4.

Gram-positive cocci

The Gram-positive cocci were the largest morphologic group recognized and were found in each subject. The majority of these isolates were acidogenic, facultative, catalase-negative, nitrate-negative, indole-negative, gelatin-negative, H_2S-negative streptococci. Representative strains formed acetic and lactic acids in PYG broth. These isolates were not *Strep. salivarius* or *Strep. mutans* as determined by colony morphology on the MST plates. Fifteen strains were studied in greater detail and the results obtained are given in Table 5. The majority of these selected strains grew in 40 per cent bile, formed NH_3 from arginine (NIVEN, SMILEY and SHERMAN, 1942), reduced methylene-blue milk forming an acid clot and fermented salicin but not inulin or mannitol. They did not agglutinate with commercially obtained streptococcal group

TABLE 4. PREDOMINANT CULTIVABLE ORGANISMS ISOLATED FROM INSTITUTIONALIZED CHILDREN

Species	Number of isolates	% Viable count	Range in % viable	Occurrence*
Gram-positive cocci	271	41·5		11/11
Streptococci sp.	247	37·8	16–66	11/11
Unidentified				
$NO_3 + H_2S^-$	15	2·3	0–10	6/11
$NO_3 - H_2S^-$	9	1·4	0–7	2/11
Gram-positive rods	232	35·3		11/11
Clostridium sp.	55	8·4	0–39	8/11
Actinomyces sp.	93	14·2	4–33	11/11
Unidentified				
$NO_3 + H_2S^+$	12	1·8	0–14	5/11
$NO_3 - H_2S^-$	25	3·8	0–21	7/11
$NO_3 - H_2S^+$	47	7·1	0–22	10/11
Gram-negative rods	111	16·9		11/11
B. melaninogenicus	37	5·6	0–22	8/11
Fusobacterium sp.	45	6·8	0–18	9/11
Unidentified				
$NO_3 - H_2S^-$	14	2·1	0–20	4/11
$NO_3^+ \; H_2S^-$	15	2·3	0–11	5/11
Gram-negative cocci	39	6		5/11
Veillonella sp.				
H_2S^+	33	5	0–32	5/11
H_2S^-	6	1	0–5	3/11
Total isolates	653	99·6		

* Number of subjects in which isolates were recovered from high dilutions of plaque.

D, E and H antisera (Difco), but did give a 2+ agglutination with MG sera. They formed an ethanol-precipitable polysaccharide in sucrose broth.

These isolates could not be readily speciated as they possessed characteristics of both *Strep. sanguis* and *Strep. mitis*. The ability to form NH_3 from arginine would

TABLE 5. CHARACTERISTICS OF SELECTED STREPTOCOCCAL ISOLATES

	Plymouth isolates	Carlsson groups*			Guggenheim biotypes†		
		V : B	IV	I : B	32	38	42
Number of strains	15	12	7	25			
Carbohydrate fermentation							
1% mannitol	0‡	0	0	0	−	−	−
1% salicin	10	10	7	25	+	+	±
1% inulin	0	1	0	24	+	−	−
Esculin hydrolysis	0	0	5	16	+	+	±
NH_3 from arginine	15	12	0	25	+	+	+
1% methylene blue milk							
Acid clot	15				+	+	−
Reduction	15				+	+	−
Growth in							
4% NaCl	0	1	0	0			
5% bile		2	3	11			
10% bile							
40% bile	11				+	−	−

* CARLSSON, J. 1968.
† GUGGENHEIM, B. 1968.
‡ Number positive.

suggest that they are similar to isolates described by CARLSSON (1968), i.e. Groups V:B and I:B and by GUGGENHEIM (1968), i.e. Biotypes 32, 38, and 42 (Table 5). However, the ability to grow in 40 per cent bile differentiates the Plymouth isolates from Group V:B, which it most nearly resembles. Other related groups are shown in Table 5.

Gram-positive rods

Two hundred and thirty-two Gram-positive rods were isolated from the eleven subjects. A significant number of isolates, i.e. 8·4 per cent, were anaerobic, Gram-positive sporulating rods and were identified as *Clostridium* species. These organisms had subterminal oval spores, were motile, nitrate-variable, H_2S-positive, gelatin-positive, indole-negative, and variable in regard to esculin hydrolysis. They were mainly nonfermentative or weakly fermentative. Acid end-product analysis of growth in PYG broth suggested that several groups were present. One group formed acetic, butyric, isovaleric, isobutyric, valeric, succinic, lactic and traces of propionic acids. Another group formed isocaproic acid among other volatile acids. The taxonomic information available was not adequate to speciate these isolates at present. However, the limited biochemical profile suggested that strains of *Cl. sporogenes, Cl. subterminale, Cl. hastiforme*, and *Cl. histolyticum* were present (VIRGINIA POLYTECH. INSTITUTE LAB., ANAEROBE LAB., 1970).

Fluorescent antibody reagents were used to confirm the presence of *Clostridium* in the plaque samples. Rabbits were immunized with several of the *Clostridium* isolates. The sera obtained were conjugated with fluorescein isothiocyanate and used to stain plaque smears made from the original plaque dilution (see Materials and Methods). Many, but not all, plaques contained organisms which gave a 3 or 4+ fluorescence with these *Clostridium* antisera.

Fourteen per cent of the isolates were Gram-positive, filamentous, branching rods suggestive of *Actinomyces* species. These organisms were present in every child. These organisms were nitrate-positive, H_2S-negative, indole-negative and gelatin-negative. They did not grow well in the CB and usually did not lower the pH below 5·5. Growth of representative strains in PYG broth was accompanied by production of acetic, lactic and succinic acids.

Seven per cent of the isolates were nitrate-negative, H_2S-positive, indole-negative, gelatin-negative, Gram-positive rods. They were either weakly fermentative or fermentative. Acid end-products formed in PYG broth included acetic, isovaleric, lactic, succinic and butyric acids.

Two other groups of Gram-positive rods could be recognized. Four per cent of the isolates were nitrate-negative, H_2S-negative, indole-negative and non-fermentative. Two per cent of the strains were nitrate-positive, H_2S-positive and weakly fermentative. These last three groups could not be speciated and presumably are diphtheroids.

The Gram-negative rods comprised 17 per cent of the cultivable flora and were found in each subject. Black pigment forming *Bacteroides melaninogenicus* averaged 5·6 per cent of the isolates. These strains were nitrate-negative, indole-variable, gelatin-positive and H_2S-positive. They were weakly to nonfermentative. These organisms were present in 8 of the 11 subjects and ranged as high as 22 per cent of the isolates in one subject. *Fusobacterium* species accounted for 6·8 per cent of the isolates. These organisms were also weakly to non-fermentative, gelatin-negative, nitrate-negative, H_2S-positive and indole-variable. These fusiforms were identified primarily by colonial and single cell morphology and the biochemical tests which were confirmatory

(LOESCHE and GIBBONS, 1965). A small group of fermentative, nitrate-negative, H_2S-negative, gelatin-negative, and indole-negative strains were found in 4 subjects and presumably were *Bacteroides* species (LOESCHE, SOCRANSKY and GIBBONS, 1964). Another group, which could not be identified, accounted for 2·3 per cent of the isolates and were nitrate-positive, H_2S-negative, gelatin-negative, indole-negative and fermentative. They were excluded from the *Bacteroides* group on the basis of being nitrate-positive.

The Gram-negative cocci were identified as *Veillonella* species and were found to comprise 6 per cent of the cultivable flora. They were present in only 5 subjects. In one subject, they were a major group accounting for 33 per cent of the isolates. These organisms were non-fermentative, nitrate-positive, H_2S-variable, gelatin-negative and indole-negative. Representative strains formed acetate and propionate when grown in PYG broth. These characteristics are compatible with the description provided by ROGOSA (1964) for the genus *Veillonella.*

DISCUSSION

The quantitative recovery of bacteria from the dental gingival surfaces is complicated by the unusual sensitivity of these organisms to oxygen. GORDON, STUTMAN and LOESCHE (1971) showed that an exposure to atmospheric oxygen of only 20–30 min during the plating of gingival plaque samples reduced the recoveries from 67 to 20 per cent. ARANKI *et al.* (1969) showed that a system of complete anaerobiosis, i.e. anaerobic chamber, tripled the recoveries of bacteria from gingival samples over that obtained with anaerobic jar procedures. In the present study, the additional problem of survival of anaerobic organisms during the 2–3 hr transport of the samples back to the laboratory had to be considered. GASTRIN, KALLINGS and MARCETIC (1968) have shown that the currently available transport media, i.e. Stuart, VMG, and SBL are inadequate for the quantitative recovery of bacteria. When 18 pure cultures of medically important bacteria were placed in these transport media, 90–99 per cent of the inocula did not survive the first hour. To overcome these difficulties, special precautions were taken in the present study to minimize exposure of the plaque to atmospheric oxygen and an oxidizing environment. Plaque samples were exposed to room atmosphere for about 10–20 sec before immersion in the DTT poised transport medium and were subsequently never exposed to oxygen. Dithiothreitol was chosen as the reducing agent because of its low redox potential (−0·33 V at pH 7), and its minimal tendency to be oxidized directly by air (CLELAND, 1964). The number of colonies which grew on the modified M10 medium accounted for 33 per cent of the cell units seen in the microscopic count (Table 2). The ratio of anaerobic to aerobic recoveries was about 4. This ratio, when anaerobic jars have been employed, is about 2 (GIBBONS *et al.*, 1963, 1964; GORDON, STUTMAN and LOESCHE, 1971). These results would indicate that the procedures employed were partially successful in overcoming the difficulties in culturing specimens comprised primarily of anaerobes.

The precautions taken to minimize oxygen exposure meant that the plaque samples were not weighed. In order to calculate the bacterial density of these plaque samples, the microscopic count per unit dry weight for plaque taken from other sites

in the same mouth at the same time was determined and a proportional equation was used to derive the weight of the cultured sample. This calculation makes the assumption that the number of bacterial units per unit plaque weight is the same in all intraoral sites and is essentially the same assumption that was made when pooled plaque samples were used (MORRIS, 1953; GIBBONS *et al.*, 1963, 1964; GORDON, STUTMAN and LOESCHE, 1971). However, site to site proportional variability may exist (POOLE and GILMOUR, 1971).

Once the toxic exposure to oxygen has been minimized, the recovery of bacteria from plaque samples, or for that matter from any specimen taken from the mucous membranes of mammals, will depend upon the availability of essential nutrients in the primary isolation medium. The ideal medium presumably would be one that provides the nutrients found in the natural habitat of the bacteria and most likely would mimic the composition of the biological fluid(s) which bathe that environment. CALDWELL and BRYANT (1966) have developed medium M10 which is based upon the composition of rumen fluid. This medium has been demonstrated to be excellent for the isolation of rumen bacteria (CALDWELL and BRYANT, 1966), anaerobic sewer sludge bacteria (MAH and SUSSMAN, 1967) and faecal bacteria (ELLER, CRABILL and BRYANT, 1971).

In the present investigation, medium M10 was modified by the deletion of starch and cellobiose and by the addition of blood, glucose, lactate, formate, nitrate and menadione (Table 1). Preliminary studies suggested that the modified M10 medium was superior to brain-heart infusion medium supplemented with blood, menadione and nitrate in the isolation of bacteria from plaque samples. For this reason modified M10 was used throughout the study.

A possible explanation as to why the modified M10 yielded excellent recoveries is that the low level of glucose will not allow fast-growing acidogenic bacteria such as the streptococci to lower the pH to levels which would inhibit the growth of slower growing organisms. However, the present results do not demonstrate its superiority over other primary isolation media in the quantitative recovery of oral plaque bacteria.

In this regard, MM10 should be compared with the nutrient broth-cysteine-haemin (N_2CH) medium shown by GILMOUR and POOLE (1970) to permit recoveries of about $11 \cdot 3 \pm 2 \cdot 5 \times 10^7$ organisms per mg plaque wet weight. The N_2CH medium is easier to prepare than the MM10 medium. In the present study, our viable counts were obtained on 2–3-hr-old samples and our microscopic counts were referred to a dry weight basis, thereby precluding a direct comparison with the data of GILMOUR and POOLE (1970). However, a calculation is possible in that the dry weight of our plaque samples accounted for about one fourth of the plaque wet weight (LOESCHE and GREEN, 1972). When the dry weight values are converted to a wet weight basis, the anaerobic viable plate count on MM10 amounts to about $10 \cdot 0 \times 10^7$/mg plaque wet weight.

The organisms recovered from plaque samples have usually been difficult to identify because of (1) incomplete descriptions in the literature and (2) the introduction of a new technique, i.e. complete anaerobiosis, usually permits recoveries of organism hithertofore undescribed. The organisms recovered in this study were no

exception. The most common isolates were streptococci strains. Colonial growth on MST showed that *Strep. mutans* and *Strep. salivarius* were present in low numbers, if at all. A small group of streptococcal strains were studied in greater detail. Their ability to form ammonia from arginine and not ferment inulin or esculin made them similar to the *Strep. mitis* found in Group V:B of CARLSSON (1968) and possibly to biotype 38 of GUGGENHEIM (1968). The Plymouth isolates were able to grow in the presence of 40 per cent bile and to form ethanol precipable polysaccharide which would distinguish these isolates from group V:B and biotype 38. Recently DE STOPPELAAR (1971) has reported on the occurrence of *Strep. sanguis* strains that grow in 40 per cent bile and produce ammonia from arginine. These considerations would indicate that the Plymouth isolates possess attributes of both *Strep. mitis* and *Strep. sanguis* and cannot be properly speciated at this time. The data do not permit the assumption that all the streptococci recovered would belong to this one group. A larger group of 42 isolates showed variability in regard to the various tests, but all strains produced ammonia from arginine. It may be that ammonia production from arginine is an important and stable characteristic of the streptococci isolated from these plaque samples. Also the relationship of these isolates to enterococci needs to be determined. The inability to detect *Strep. mutans* in high numbers in the 11 subjects could be a reason for the low caries experience found in these individuals.

The demonstration of *Clostridium* species in these plaque samples was unexpected. Previous cultural studies of plaque taken from non-institutionalized subjects (MORRIS, 1953; GIBBONS *et al.*, 1963, 1964; HOWELL, RIZZO and PAUL, 1965; ARANKI *et al.*, 1969) did not disclose the presence of *Clostridium*. Also investigations of samples taken from periodontally diseased pockets did not show clostridia either by darkfield examinations (ROSEBURY, MACDONALD and CLARK, 1950) or by cultural methods (DWYER and SOCRANSKY, 1968; MCMINN and CRAWFORD, 1970). Recently the presence of clostridia in periodontal debris has been reported (VAN REENEN and COOGAN, 1970) and *Clostridium welchii* has been isolated from carious dentine (HARTLES and MCDONALD, 1951). However, in these two studies, a high dilution technique was not used, and the clostridia eventually isolated could have been present in low numbers or actually been transient in nature. The present findings show that *Clostridium* species comprised about 8 per cent of the total cultivable flora and therefore are numerically prominent in the tooth surface plaque of these institutionalized subjects. The presence of these organisms in plaque was confirmed by fluorescent antibody reagents. The Plymouth subjects have ample exposure to soil and faecal contamination as they spend considerable time on the floor and in the lavatories. Thus it may not be surprising that in this population, clostridia can be isolated from the oral cavity. Whether the presence of clostridia contributes to the early breakdown of periodontal tissues that is common in institutionalized mentally retarded individuals (SWALLOW, 1964, CUTRESS, 1971a, 1971b) remains to be demonstrated. In this regard, the possible presence of *Cl. histolyticum* in plaque samples may be of clinical significance as this organism possesses a potent extracellular collagenase (SMITH and HOLDEMAN, 1968).

The organisms classified as *Actinomyces* species need additional study. They were second only to the streptococci in prevalence and in numerical importance. Their

biochemical characteristics, with one exception, were compatible with these isolates being strains of *Actinomyces israeli, Act. naeslundii, Act. viscosus* or *Act. odontolyticus* (SMITH and HOLDEMAN, 1968). The exception was the inability of our isolates to acidify the CB medium.

Bacteroides melaninogenicus was a conspicuous organism in these plaque samples. This contrasts with the findings of GIBBONS *et al.* (1964) where *B. melaninogenicus* was not detected in tooth surface plaque taken from normal individuals. MESKIN, FARSHT and ANDERSON (1968) reported an increased prevalence of *B. melaninogenicus* in institutionalized subjects, which would be in agreement with our findings. However, CUTRESS, BROWN and GUY (1970) found *B. melaninogenicus* in their sampling of institutionalized children to account for less than 0·2 per cent of the total viable count. These workers might have lost *B. melaninogenicus* during the transport and aerobic manipulation of their samples.

On the basis of this partial characterization of the supragingival plaque isolates from the Plymouth subjects, it would seem that this flora differed from the supragingival plaque isolates recovered by GIBBONS *et al.* (1964) from normal subjects. *B. melaninogenicus, Fusobacterium* species and *Clostridium* species were found in the Plymouth subjects but not in the plaque of normal subjects. Even spirochaetes were present on darkfield examination prior to dispersal of the sample. (The failure of the spirochaetes to grow on medium MM10 might reflect their lysing during the manipulation of the sample, that their nutritional needs were not met, or that they were present in numbers below our dilution range.) These observations suggest that the tooth surfaces in these individuals have been colonized by certain bacteria, with the exception of the *Clostridium* species, which apparently are restricted to the gingival crevice area in normal subjects (SOCRANSKY *et al.*, 1963). The presence of *Clostridium* species may be unique to this population and might reflect the easy access institutionalized subjects have to soil and faecal contamination.

As the clinical situation between normal individuals and mentally retarded institutionalized subjects are dissimilar (CUTRESS, 1971a, 1971b), some speculation as to the role the different isolates play in these clinical conditions is possible. However, the extrapolation of *in-vitro* pure culture results to the *in-vivo* condition must be considered as mainly setting up hypothesis for future studies, as the *in-vitro* and *in-vivo* metabolism of bacteria need not be identical (GORDON and GIBBONS, 1967).

The quantitative culturing of tooth surface plaque from normal subjects showed that about 84 per cent of the cultivable isolates fermented glucose (calculated from the data of GIBBONS *et al.*, 1964) and that the overwhelming majority of isolates in early plaque are acidogenic streptococci (THEILADE and THEILADE, 1969). The plaque from the Plymouth subjects had by comparison only 50 per cent of the cultivable isolates lowering the pH in glucose broth below 5·5. Also, the streptococcal strains which comprised 38 per cent of the total Plymouth isolates and 76 per cent of the acidogenic isolates were distinctive in their ability to liberate ammonia from arginine. This might mean that, over a pH range of 5·0–8·5 (NIVEN, SMILEY and SHERMAN, 1942), these organisms, if arginine was present in their environment, could be contributing base equivalents to neutralize any acid which they produced. Thus, *in vivo*

these streptococci may not contribute to any appreciable pH drop. The streptococci plus the high percentage of ammonia-producing species such as *B. melaninogenicus*, *Fusobacterium* sp. and the various *Clostridium* species would not be expected to produce a plaque capable of decalcifying enamel. Thus the bacteriological findings would seem to offer an explanation as to why the caries prevalence was so low in the Plymouth subjects.

The results of this study permit comment on the relationship between *Strep. sanguis* and caries. Many *Strep. sanguis* strains produce ammonia from arginine (CARLSSON, 1968; GUGGENHEIM, 1968; DE STOPPELAAR, 1971; THOMSON, 1971). Also *Strep. sanguis* has not been associated with extensive caries in experimental animals (FITZGERALD, 1968, KRASSE and CARLSSON, 1970) nor related to smooth surface cavities in man (DE STOPPELAAR, VAN HOUTE and BACKER DIRKS, 1969). In fact, a tendency toward a negative correlation between the presence of *Strep. sanguis* and dental caries in man (DE STOPPELAAR, VAN HOUTE and BACKER DIRKS, 1969) and in monkeys (BOWEN, 1965) has been reported. It may be that in *Strep. sanguis* the inability to cause caries and the ability to form ammonia from arginine are casually related. At pHs known to occur in plaque, these *Strep. sanguis* strains could be releasing two equivalents of a strong base which might effectively neutralize any acid in their immediate environment. Thus a plaque in which *Strep. sanguis* is the main streptococcal species may not *in vivo* be capable of achieving the acid pH necessary to dissolve the enamel surface.

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Résumé—Des plaques de surfaces dentaires, prélevées chez 11 sujets, vivant en institution, sont mises en culture sur agar en chambre anaérobie. La récupération des bactéries dans un milieu anaérobie dilué d'extrait trypticase levure (MM 10) est d'environ 33 ± 26 pour cent de la numération microscopique. La croissance sur MM 10, dans des conditions anaérobies, avoisine 8 ± 5 pour cent. Le rapport de récupération des bactéries anaérobies par rapport aux aérobies sur MM 10 est d'environ 4. Six cent soixante et onze isolements ont donné des subcultures et ont pu être caractérisés partiellement. Environ la moitié des isolements ont été incapables d'abaisser le pH dans un bouillon de glucose en dessous de 5,5. Les streptocoques en constituent environ 38 pour cent et ont été retrouvés chez chaque sujet. Un sous-groupe de 15 souches a poussé sur 40 pour cent de bile, a formé du NH_3 à partir de l'arginine et a fermenté la salicine, mais non l'inuline. Ces organismes présentent les caractéristiques du *Strep. sanguis* et du *Strep. mitis*. Diverses espèces *d'Actinomyces* constituent 14 pour cent et les *Clostridium* forment 8 pour cent de la flore cultivable. *Bacteroides melaninogenicus*, *Fusobacterium* et *Veillonella* constituent chacun environ 6 pour cent des isolements. Le caractère général de ces plaques semble indiquer qu'une flore microbienne du sillon gingival, contenant plusieurs espèces susceptibles de fermenter les acides aminés, a colonisé les surfaces dentaires. Ces micro-organismes ne sont pas susceptibles de produire une plaque capable de décalcifier l'émail, ce qui pourrait expliquer la faible fréquence carieuse de ces sujets.

Zusammenfassung—Plaques wurde von den Zahnoberflächen von 11 institutionalisierten Personen entnommen und auf Agarplatten anaerob kultiviert. Die Wiedergewinnung der Mikroorganismen in einem anaerob inkubierten löslichen Tryptikase-Hefe-Extrakt-Medium (MM 10) entsprach durchschnittlich 33 ± 26 Prozent der mikroskopischen Zählung. Das Wachstum auf MM 10 betrug unter aeroben Bedingungen durchschnittlich 8 ± 5 Prozent. Das anaerob zu aerobe Verhältnis der Wiedergewinnung auf Medium MM 10 betrug etwa 4. 671 Isolate wuchsen auf Subkulturen und wurden teilweise charakterisiert. Etwa die Hälfte der Isolate war nicht dazu fähig, das pH in Glukosebrühe unter 5,5 zu senken. Streptokokken machten etwa 38 Prozent der Isolate aus und wurden bei jeder Person gefunden. Eine Untergruppe von 15 Stämmen wuchs in 40 Prozent Galle, bildete NH_3 aus Arginin und fermentierte Salizin, jedoch nicht Inulin. Diese Isolate wiesen Charakteristika von *Strep. sanguis* und *Strep. mitis* auf. Verschiedene *Actinomyces*-species umfaßten etwa 14 Prozent, und *Clostridium*-species machten 8 Prozent der kultivierbaren Flora aus. *Bacteroides melaninogenicus*, *Fusobacterium*-Arten und *Veillonella*-Arten waren jeweils in etwa 6 Prozent der Isolate vorhanden. Der Gesamtcharakter dieser Plaque-Isolate legt es nahe, daß die Mikroflora der Zahnfleischfurche, welche mehrere Aminosäure fermentierende Arten enthält, auf den Zahnoberflächen aufgewachsen waren. Es ist nicht zu erwarten, daß diese Mikroorganismen Plaques entwickeln, die den Schmelz zu entkalken vermögen; daraus dürfte sich erklären, warum diese Personen wenig Karies hatten.

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Antigenic Heterogeneity of *Bacteroides intermedius* as Recognized by Monoclonal Antibodies

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Four hybrid cell lines secreting monoclonal antibodies against antigens of *Bacteroides intermedius* were generated by fusing murine NSI cells with splenocytes from a rat immunized with *B. intermedius* strain OMZ248. An enzyme-linked immunosorbent assay was used to analyze the distribution of the recognized antigens on 39 strains from various *Bacteroides* species and on 5 strains from other genera. Only *Bacteroides* species *B. intermedius*, *B. loescheii*, *B. melaninogenicus*, and *B. corporis* were found to express at least one of the recognized antigens. Strains of the two asaccharolytic black-pigmenting *Bacteroides* species were negative. Among the strains capable of binding to one or more of the monoclonal antibodies, five groups with different reactivity patterns could be distinguished. Two of the monoclonal antibodies were specific for *B. intermedius*. The *B. intermedius* strains were metabolically almost identical, expressed at least three of the recognized antigens, and fell into three distinct antibody reactivity groups, suggesting a tentative separation of this species into three new serogroups. Oral and nonoral isolates of *B. intermedius* were, however, not distinguished by the monoclonal antibodies. One monoclonal antibody was directed against an antigen strongly expressed on all saccharolytic black-pigmenting *Bacteroides* strains tested so far, thus confirming the previously noted antigenic relationship between the species which had emerged from the former *B. melaninogenicus* subsp. *intermedius* and *B. melaninogenicus* subsp. *melaninogenicus* groups.

Evidence indicating a major role of bacteria in the etiology of periodontal disease is convincing. However, the microbial flora associated with gingivitis and periodontitis is complex and of considerable variation from patient to patient as well as from site to site. Furthermore, various sites of patients with different forms of periodontal disease, such as localized juvenile periodontitis or adult periodontitis, appear to harbor characteristically different sets of microorganisms (18, 21, 23, 25). It is only from recent work that information about the composition of the microflora of active sites of patients with a given type of periodontal disease has begun to emerge (12; A. C. R. Tanner and S. S. Socransky, J. Dent. Res. vol. 62, abstr. no. 350, 1983; S. S. Socransky et al., J. Dent. Res. vol. 62, abstr. no. 250, 1983). Among the bacteria encountered the most frequently in periodontally diseased sites are black-pigmenting *Bacteroides* species. Particularly, the two species *B. gingivalis* and *B. intermedius* proliferate very significantly during progressive gingival pocket formation and alveolar bone destruction (14, 21–23, 28, 29). The specific role, if any, of these two species in the development of the disease is, however, still unknown.

A widely used approach to assess the etiological importance of distinct oral microorganisms in periodontal disease is the analysis of the specific humoral immune response in both patients and healthy control persons. So far, this type of analysis has indicated a strong correlation of high immunoglobulin G (IgG) and IgA titers against *Actinobacillus actinomycetemcomitans* with the occurrence of localized juvenile periodontitis (3, 6, 24) and against *B. gingivalis* with rapidly progressive and adult periodontitis (6, 16, 24). On the other hand, specific antibody titers against *B. intermedius* have been reported to be high in most individuals regardless of their periodontal state of health (6, 19). For several reasons, the latter data cannot presently be properly interpreted. (i) The habitat of *B. intermedius* is not restricted to the oral cavity. If oral and nonoral forms of *B. intermedius* are antigenically indistinguishable as suggested by several studies (11, 17, 20), then nonoral infections or abscess formations in control individuals could indirectly prevent detection of specific immune

responses to oral *B. intermedius* infections in patients with periodontal disease. (ii) Different saccharolytic black-pigmenting *Bacteroides* species seem to exhibit considerable immunological cross-reactivity, making even the experimental production of high-titer species-specific rabbit antisera a difficult task (11, 17, 20). (iii) Different isolates of *B. intermedius* appear to be antigenetically quite heterogeneous. The heterogeneity may be such that differences in the reactivity of a given human serum with several *B. intermedius* strains may exceed the titer variations observed when several sera are tested against a single isolate (Gmür, unpublished data).

Clearly, for a better understanding of the relevance of serological reactions against *B. intermedius*, more information about the antigenic properties of *B. intermedius* as well as of other black-pigmenting species is necessary.

In this paper, we report the production of four monoclonal antibodies against *B. intermedius* strain OMZ248 and describe the distribution of the recognized antigens on 28 *B. intermedius* isolates and on 16 strains from other species. The data emphasize the antigenic heterogeneity of *B. intermedius*, but also confirm the previously noted antigenic relationship among saccharolytic black-pigmenting *Bacteroides* species.

MATERIALS AND METHODS

Bacterial strains. Of the 44 strains used in this study, 38 were black- or brown-pigmenting *Bacteroides* strains of human origin. Seventeen strains, designated herein as reference strains, have been described previously and are listed in Table 1. Twenty-seven strains were isolated in this laboratory from periodontal pockets (>4-mm pocket depth) of 27 patients.

Preliminary biochemical tests done before this study identified 24 of these strains as *B. intermedius*, 2 as belonging to the former *B. melaninogenicus* subsp. *melaninogenicus* group, and 1 (strain OMZ274) as *Fusobacterium nucleatum*. All strains were stored in liquid nitrogen and also were lyophylized.

Culture conditions and biochemical tests. Wilkins-Chalgren anaerobe agar (Oxoid Ltd.) supplemented with 5% (vol/vol) hemolyzed human blood was used as solid medium. All strains were able to grow in a modification of a fluid medium (FUM) originally described by Loesche et al. (13). FUM contained (per liter of distilled water): 10 g of tryptone, 5 g of yeast extract, 3 g of glucose, 2 mg of hemin, 1 mg of menadione, 0.5 g of cysteine hydrochloride, 0.1 g of dithiothreitol, 2.9 g of NaCl, 0.5 g of Na_2CO_3, 1 g of KNO_3, 0.45 g of K_2HPO_4, 0.45 g of KH_2PO_4, 0.9 g of $(NH_4)_2SO_4$, and 0.188 g of $MgSO_4 \cdot 7H_2O$ and had a pH of 7.1. Heat-inactivated, filter-sterilized horse serum was finally added to this medium, which was prepared and sterilized as originally described (13). All cultures were incubated anaerobically in jars (BBL Microbiology Systems) at 37°C for 3 to 5 days.

Gram stains, catalase activity tests, and growth experiments in air gave negative results for all black-pigmenting strains. In addition, the strains were characterized as follows.

Fermentation of glucose, lactose, mannitol, and trehalose was tested in glucose-free FUM containing 1% of the respective filter-sterilized compounds. Cultures in FUM were additionally analyzed for indole production. In later experiments performed to reevaluate the indole production of several strains, a carbohydrate- and KNO_3-free FUM medium was used. Esculin hydrolysis was tested in FUM containing 1% esculin. The production of lipase and lecithinase was tested by standard procedures (27), using Wilkins-Chalgren agar as a base for the egg yolk supplement. Milk proteolysis was examined on Wilkins-Chalgren agar plates containing 5% skim milk powder; 5 g was suspended in 100 ml of water, sterilized by repeated heating in flowing steam, and added to 900 ml of precooled (57°C) medium. Finally, the capacity of these strains to hydrolyze soluble starch and purified gelatin (E. Merck AG) was tested by adding 1% of the respective compounds to the same medium.

Identification of fermentation products. The bacteria were incubated in FUM with 1% glucose for 40 to 120 h. The cultures were then centrifuged, and the supernatants were acidified with 4 N HCl to pH 2.0. A 1-ml volume of diethyl ether was thoroughly mixed with a 2-ml sample of the acidified culture fluid. The organic phase was separated from the water phase by freezing (−20°C) and was then analyzed by gas-liquid chromatography (2).

Cell lines. The murine myeloma cell line NSI-AG 4/1 (10) was provided by B. K. Grove, Institute for Cell Biology, Swiss Federal Institute of Technology, Zurich, Switzerland. The cells were grown in Dulbecco minimal essential medium supplemented with 10% heat-inactivated newborn bovine serum (GIBCO Europe), 15 μg of 8-azaguanine per ml, 2 mM glutamine, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. The isolation and cultivation of diploid fetal rat fibroblasts have been described previously (5).

Production of hybrid cells. An inbred RIC (rat inbred at Carworth Farm, New York, N.Y.)-Sprague-Dawley rat was subcutaneously immunized with approximately 10^8 bacteria of *B. intermedius* OMZ248, emulsified in 0.1 ml of saline and an equal volume of incomplete Freund adjuvant. Intraperitoneal booster injections with 100 μg of a lyophilized broken-cell supernatant (1) of strain OMZ248 bacteria were administered after 4 and 9 months. Three days after the last injection, the rat was sacrificed, and splenocytes were isolated by gently pressing the spleen through a stainless steel mesh in Ca^{2+}- and Mg^{2+}-free Dulbecco phosphate-buffered saline. Erythrocytes were lysed by hypotonic shock with 0.83% NH_4Cl, and 9×10^7 spleen cells were fused with 9×10^6 NSI cells as described by Gmür et al. (7). After fusion, the cell suspension was plated in 10 24-well tissue culture plates (Nunc) in selection medium that consisted of Iscove-modified minimal essential medium (KC Biologicals Inc.) supplemented with 10% heat-inactivated newborn bovine serum, 13.6 μg of hypoxanthine per ml, 1.76 μg of aminopterin per ml, 3.9 μg of thymidine per ml, 0.225 μg of glycine per ml, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 1% (vol/vol) anti-PPLO agent (GIBCO Europe). Wells with growth of cell hybrids were tested for the presence of antibodies

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TABLE 1. Reference strains used in this study

Species and strain	Site of origin	Received from[a]:
B. asaccharolyticus		
ATCC25260 (VPI4198)	Empyema	T. J. M. van Steenbergen
NCTC9337 (VPI8945)	Infected hemorrhoids	G. H. Bowden
B. gingivalis		
381	Subgingival plaque	S. S. Socransky
1021	Subgingival plaque	J. Slots
W1 (Loesche W)	Subgingival plaque	N. P. Lang
B. intermedius		
Nonoral		
ATCC25261 (VPI4203)	Laryngotomy wound	T. J. M. van Steenbergen
ATCC25611 (VPI4197)	Empyema	T. J. M. van Steenbergen
Oral		
H187	Subgingival plaque	T. J. M. van Steenbergen
M107-74 (VPI9146)	Subgingival plaque	G. H. Bowden
B. corporis		
532-70A (ATCC33547, VPI9342)	Cervical swab	T. J. M. van Steenbergen
B. melaninogenicus		
VPI9343	Great toe	T. J. M. van Steenbergen
B. loescheii		
ATCC15930 (VPI0037)	Gingival crevice	T. J. M. van Steenbergen
B. fragilis		
ATCC25285	Appendix abscess	J. Wüst
F. nucleatum		
OMZ274	Subgingival plaque	Our isolate
A. actinomycetemcomitans		
Y-4	Subgingival plaque	J. Slots
A. viscosus		
T14V	Supragingival plaque	B. F. Hammond
Nyl	Rat plaque	J. S. van der Hoeven
S. mutans		
OMZ176	Supragingival plaque	Our isolate

[a] T. J. M. van Steenbergen, Free University, Amsterdam, The Netherlands; G. H. Bowden, University of Manitoba, Winnipeg, Canada; S. S. Socransky, Forsyth Dental Center, Boston, Mass.; J. Slots, State University at Buffalo, Buffalo, N. Y.; N. P. Lang, University of Berne, Switzerland; J. S. van der Hoeven, University of Nijmegen, The Netherlands; J. Wüst, University of Zurich, Switzerland; B. F. Hammond, University of Pennsylvania, Philadelphia.

against *B. intermedius* OMZ248 on days 11 and 17 by an enzyme-linked immunosorbent assay (ELISA) as described below. To prevent overgrowth by nonproducer cells, wells with apparent specific antibody production were split at low density, whereby one part of the cells was used for cloning by limiting dilution in 96-well tissue culture plates (Nunc) and the other part was transferred into new plates and expanded for freezing. Most of the initial splits failed to yield clones because of rather low plating efficiencies of the hybrid cells. This problem was overcome by propagating all cultures on feeder layers of irradiated (2,000 rad) diploid fetal rat fibroblasts.

The fusion finally yielded four independent hybrid cell lines producing antibodies against *B. intermedius*. The four lines were cloned by limiting dilution, expanded to mass cultures, and then immediately recloned again. After being cloned once, all cultures were switched to selection medium lacking aminopterin (HT medium). All experiments described herein were done with pools of culture supernatant harvested from fibroblast feeder layer-supported mass cultures of clonal hybridoma lines.

For isotyping of monoclonal antibodies, undiluted culture supernatants were tested with rat immunoglobulin class-specific reagents (Nordic Immunological Laboratories) in Ouchterlony gel diffusion.

ELISA. Both the screening of hybridoma culture

fluids for the presence of antibodies against *B. intermedius* and the analysis of the strain distribution of the antigens recognized by the isolated monoclonal reagents were done with a modified ELISA (4). Round-bottom 96-well microtest plates (Greiner) were coated with antigen by adding 100 μl of bacterial broken-cell supernatant per well (10 μg of protein per ml of phosphate-buffered saline) and incubating the plates overnight at 4°C. The wells were then washed twice with borate-buffered saline (BS; 0.14 M boric acid, 0.025 M sodium tetraborate, 0.075 M sodium chloride, pH 8.1), refilled with 100 μl of 0.5% bovine serum albumin (BSA) and 0.05% Tween 20 per well in BS and allowed to stand for 2 h at 37°C. After being washed twice with BS and once with distilled water, the wells were dried over water-free calcium chloride. At that point, the plates were sealed tightly and transferred to 4°C so that they could be stored for several months without loss of antigenic activity. Alternatively, bacteria were grown in fluid cultures for 24 to 72 h, washed twice with 0.9% sodium chloride, suspended in PBS, and diluted to a standard optical density of 0.5 at 550 nm. Flat-bottom ELISA microtest plates (Inotech) which had been pretreated with 100 μl of 0.1% glutaraldehyde per well in BS for 30 min at room temperature were filled with 200 μl of bacterial suspension per well and centrifuged (10 min at 1,300 × *g*). Without decantation, an additional 100 μl of 0.1% glutaraldehyde in BS was then added carefully to each well, and the plates were allowed to stand for 30 min at room temperature. After two washing steps with BS, the wells were filled with 200 μl of 0.1% BSA per well in BS, and the plates were sealed tightly and stored at 4°C until used. No loss of antigenic activity was observed for as long as 12 months.

The antigen-antibody binding assay procedure involved three steps. (i) Antigen-coated microtest plates were washed twice with BS and incubated overnight at 4°C with monoclonal reagent or antisera that had been diluted with BS containing 0.5% BSA and 0.05% Tween 20. Unbound antibodies were then removed by flicking the plates, and wells were washed four times with ELISA wash solution (0.9% sodium chloride, 0.05% Tween 20, 0.01% sodium azide). (ii) a 100-μl volume of horseradish peroxidase-conjugated rabbit anti-rat IgG per well, prepared by the method of Miller et al. (15) and diluted 1/2,000 in BS containing 0.5% BSA and 0.05% Tween 20, was added to each microtest well. The plates were incubated for 90 min at 37°C, and the wells were washed eight times with ELISA wash solution before being refilled (step iii) with 200 μl of enzyme substrate solution (3.7 mM 1,2-phenylenediamine, 0.012% hydrogen peroxide in 24.3 mM citric acid, 25.7 mM disodium hydrogen phosphate, pH 5.1). After a 15-min incubation at room temperature in the dark, the enzyme reaction was stopped by addition of 50 μl of 2.5 M sulfuric acid per well, and the extent of the reaction was determined spectrophotometrically at 492 mm by using a Titertek Multiscan (Flow Laboratories Ltd.).

Screening of cell hybrid culture fluids. Fluids from cultures with proliferating hybrid cells were tested with ELISA for the production of antibodies against *B. intermedius* by adding 50 μl (cloning cultures) or 100 μl (mass cultures) to individual wells of both microtest plates coated with strain OMZ248 broken-cell supernatant and to plates coated with native strain OMZ248 bacteria from fluid cultures. Positive control wells contained 1/500-diluted serum obtained from the rat whose spleen was used for hybridization.

Analysis of strain distribution of the antigens recognized by monoclonal antib dies. Analysis was done by (i) directly measuring with ELISA the binding of the monoclonal reagents to bacteria of various strains and by (ii) quantitative absorption of the monoclonal reagents with various strains.

In the first analysis procedure, the wells of eight horizontal rows of ELISA microtest plates were coated with suspensions of eight different bacterial strains. All suspensions were adjusted to identical optical densities (0.5 at 550 nm). For reference purposes, the wells of one row always contained strain OMZ248 bacteria. The wells of each row were then filled with serial dilutions of one of the monoclonal reagents and processed by the ELISA procedure outlined above.

In the second analysis procedure, bacteria of a given strain were harvested from at least two 14-ml fluid cultures, pooled, and quantitated by measuring the optical density at 550 nm of a threefold-diluted sample. The bacteria were then washed twice with phosphate-buffered saline and centrifuged again, and the pellets were suspended in an amount of HT medium calculated by the formula $y = az$, where y is the amount (milliliters) of HT medium used for the suspension of the pellet, z is the previously determined optical density, and a is a dilution factor. The concentration of bacteria in a suspension obtained with $a = 1.67$ was arbitrarily defined as 1×. It was determined in a preliminary experiment (data not shown) that the 1× concentration equals that obtained when 100 μl of centrifugation-sedimented bacteria was suspended in 2 ml of HT medium. Absorptions of monoclonal reagents were always performed with at least three different concentrations of bacteria. To this end, 100 μl of bacterial suspensions (either 1×, 1/5× and 1/25× or 4×, 2×, 1×, 1/2× and 1/4× depending on the experiment) were mixed in 96-well round-bottom microtest plates with 50 μl of monoclonal reagent. The dilution of the monoclonal reagent was chosen such that the unabsorbed reagent yielded 70 to 90% of maximal binding in ELISA with strain OMZ248 bacteria (optical density, 0.3 at 550 nm) as antigen. The plates were then tightly sealed and incubated at 37°C on a shaker. After 2 h, the plates were centrifuged (1,300 × *g* for 10 min), 100 μl of the supernatant from each well was transferred into wells of ELISA microtest plates coated with strain OMZ248 bacteria (optical density, 0.3 at 550 nm), and ELISA was performed as described above.

RESULTS

Isolation of hybrid cell lines producing monoclonal antibodies against *B. intermedius*. Culture supernatants from 130 wells with growing hybrid colonies were screened in ELISA for activity against intact bacteria and broken-cell supernatants of strain OMZ248. Nine supernatants were found to be strongly positive. From four cell populations, hybrid cell lines producing antibodies against *B. intermedius* could be isolated and cloned. The four clonal cell lines and the antibodies produced by them were named 37BI6.1, 38BI1, 39BI1.1, and 40BI3.2, where the first

numbers identify the original cell line, BI is the immunogen used, and the last numbers indicate the clone obtained from the original isolate.

The supernatants from all four hybridoma lines reacted with both strain OMZ248 broken-cell supernatant and OMZ248 bacteria, but to very different extents. With the supernatant as the antigen source, 38BI1 yielded a strong reaction and a 50% endpoint titer of 1:320, whereas the supernatants of 39BI1.1 and 40BI3.2 were of lower titers and produced much less intense ELISA reactions. Monoclonal antibody 37BI6.1 bound only in barely detectable amounts to broken-cell supernatant-coated ELISA wells. In marked contrast, all four monoclonal antibodies were found to react strongly with intact strain OMZ248 bacteria (Fig. 1).

In Ouchterlony gel diffusion tests, the monoclonal antibodies 38BI1, 39BI1.1, and 40BI3.2 were all identified as IgG2a, whereas 37BI6.1 was IgG2b.

Analysis of strain distribution of the antigens recognized by monoclonal antibodies. Figure 1 shows the binding pattern of the monoclonal antibodies with the four *B. intermedius* strains ATCC25611, ATCC25261, H187, OMZ248, and with *B. corporis* 532-70A. Only two of these strains, the nonoral isolate ATCC25611 and the oral strain OMZ248, were able to bind all four antibodies. At high antibody concentrations, binding reached plateau levels, and 50% endpoint titers were between 1/100 (37BI6.1) and 1/4,400 (40BI3.2). Two other strains, ATCC25261 and H187, bound three of the monoclonal antibodies but did not react with 40BI3.2. Interestingly, these two strains could be distinguished by their reactivities with antibody 39BI1.1. Whereas strain ATCC25611 showed the same type of dose-response curve with 39BI1.1 as did strain ATCC25261 or OMZ248, the strain H187 bacteria could only be labeled with a 1,000-fold-higher antibody concentration. Furthermore, the dose-response curve never reached plateau levels (Fig. 1c). Strain 532-70A, the type strain of *B. corporis* (9), reacted only with 38BI1. Taken together, the four *B. intermedius* strains revealed as many as three different reactivity patterns with the monoclonal antibodies but also shared one of the recognized antigens with the *B. corporis* type strain.

These results prompted us to study a larger panel of *B. intermedius* strains and, to further analyze the species specificity of the monoclonal antibodies, to include a variety of strains from other *Bacteroides* species and from other genera in the analysis. Results from these experiments are summarized in Fig. 2. Of the 28 *B. intermedius* strains tested, 16 (57%) reacted with all four monoclonal antibodies, whereas 12 (43%) failed to bind antibody 40BI3.2. The latter group of strains further separated into two subgroups of seven (25%) and five (18%) strains, respectively. The 16 strains of the first group and the 7 strains falling into the larger subgroup of the second group exhibited the same type of dose-response curve with 39BI1.1 as did strain ATCC25611 or OMZ248 (Fig. 1c). All five strains of the smaller subgroup, however, revealed dose-response curves nearly identical to the one described in Fig. 1c for strain H187.

Unable to react with the four antibodies were the two *B. asaccharolyticus* strains tested, as well as all three strains of *B. gingivalis*. Also negative were all strains analyzed of the four genera other than *Bacteroides*.

Finally, four strains showed the same reactivity patterns as that of *B. corporis* 532-70A in that they bound only monoclonal antibody 38BI1. Note that all four were black-pigmenting *Bacteroides* strains positive for lactose fermentation and esculin hydrolysis and negative for indole production (see Table 5). By these criteria, they belong neither to *B. intermedius*, *B. asaccharolyticus*, *B. gingivalis*, nor *B. corporis*.

Characterization of strain heterogeneity by absorption analysis. In the course of the experiments summarized in Fig. 2, we observed that a few strains were coating the wells of ELISA plates less efficiently than others. Particularly, strain 532-70A appeared to barely adsorb to the wells. We therefore reinvestigated the results shown in Fig. 2 by absorption analysis. Increasing numbers of bacteria from representative strains were incubated with adequately diluted monoclonal reagents, and the absorbed reagents were subsequently tested on strain OMZ248 bacteria for remaining activity. The results are shown in Table 2; for ease of comparison, only absorption data from one concentration of bacteria are shown. For strains belonging to the three largest groups observed in Fig. 2, i.e., the reactivity groups including strains ATCC25611, OMZ245, and ATCC25260, respectively, the absorption experiments confirmed the results shown above. The strains of *B. intermedius* forming the subgroup containing strain H187 (Fig. 2), however, were unable to absorb 39BI1.1, in spite of the fact that these strains could bind this antibody in direct tests. This result may suggest that these strains express a slightly cross-reactive antigen. Finally, the five strains that in direct ELISA-binding tests were recognized by 38BI1 only surprisingly split into two subgroups. The three strains OMZ254, OMZ255, and ATCC15930 quite clearly absorbed antibody 37BI6.1 at the highest bacterial concentration used for absorption (1×), but did not at 1/5× and 1/25× concentrations. That this absorption is not due to unspecific antibody

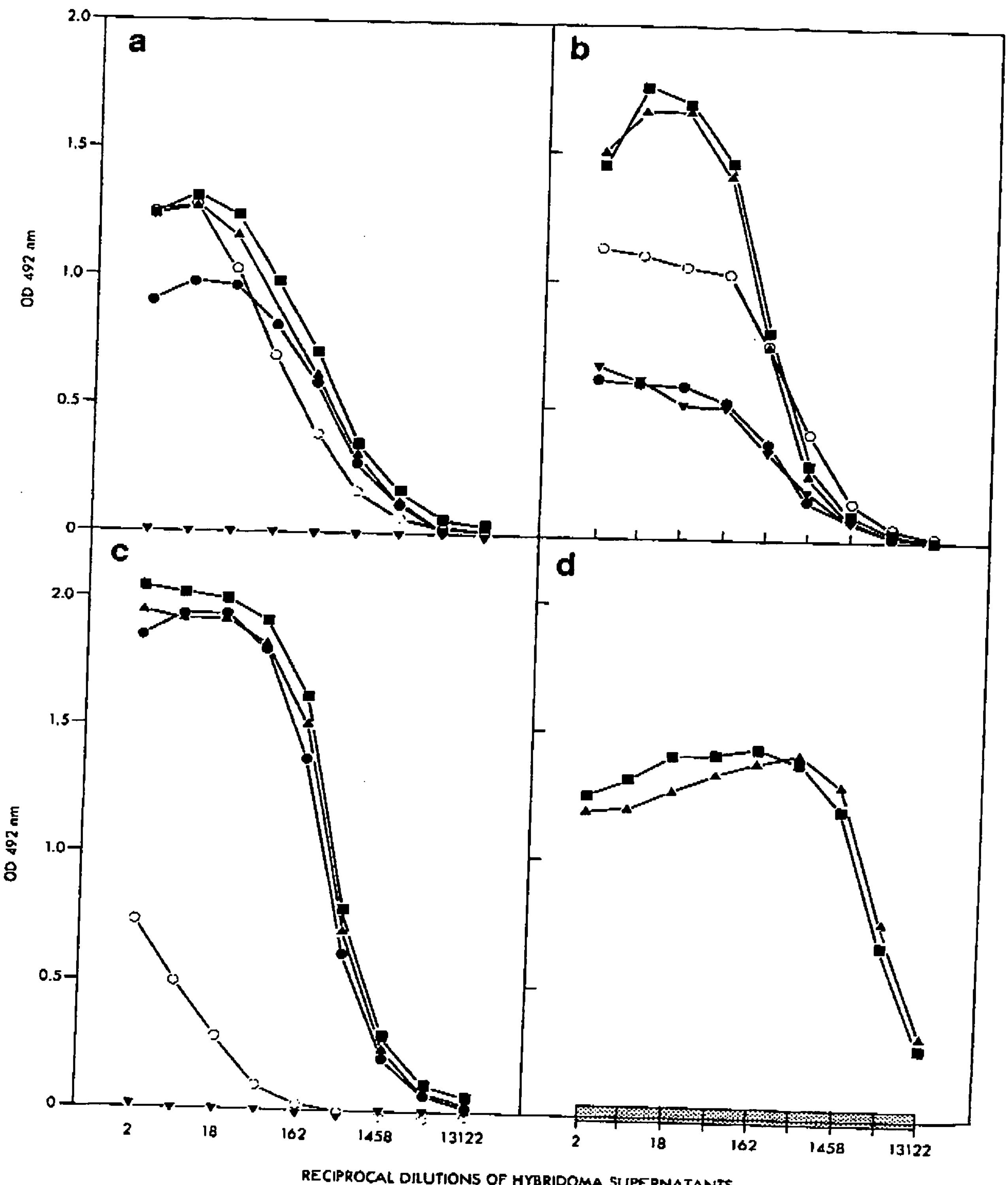
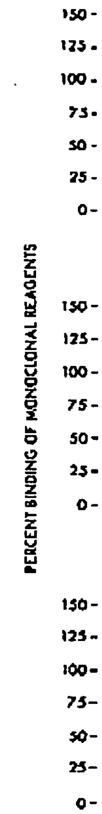


FIG. 1. Binding of the four monoclonal antibodies (a) 37BI6.1, (b) 38BI1, (c) 39BI1.1, and (d) 40BI3.2 to the four *B. intermedius* strains OMZ248 (■), ATCC25611 (▲), ATCC25261 ( ), and H187 (○) and to *B. corporis* 532-70A (▼). Binding was measured by ELISA with intact bacteria coated to 96-well microtest plates. OD 492 nm, Optical density at 492 nm.



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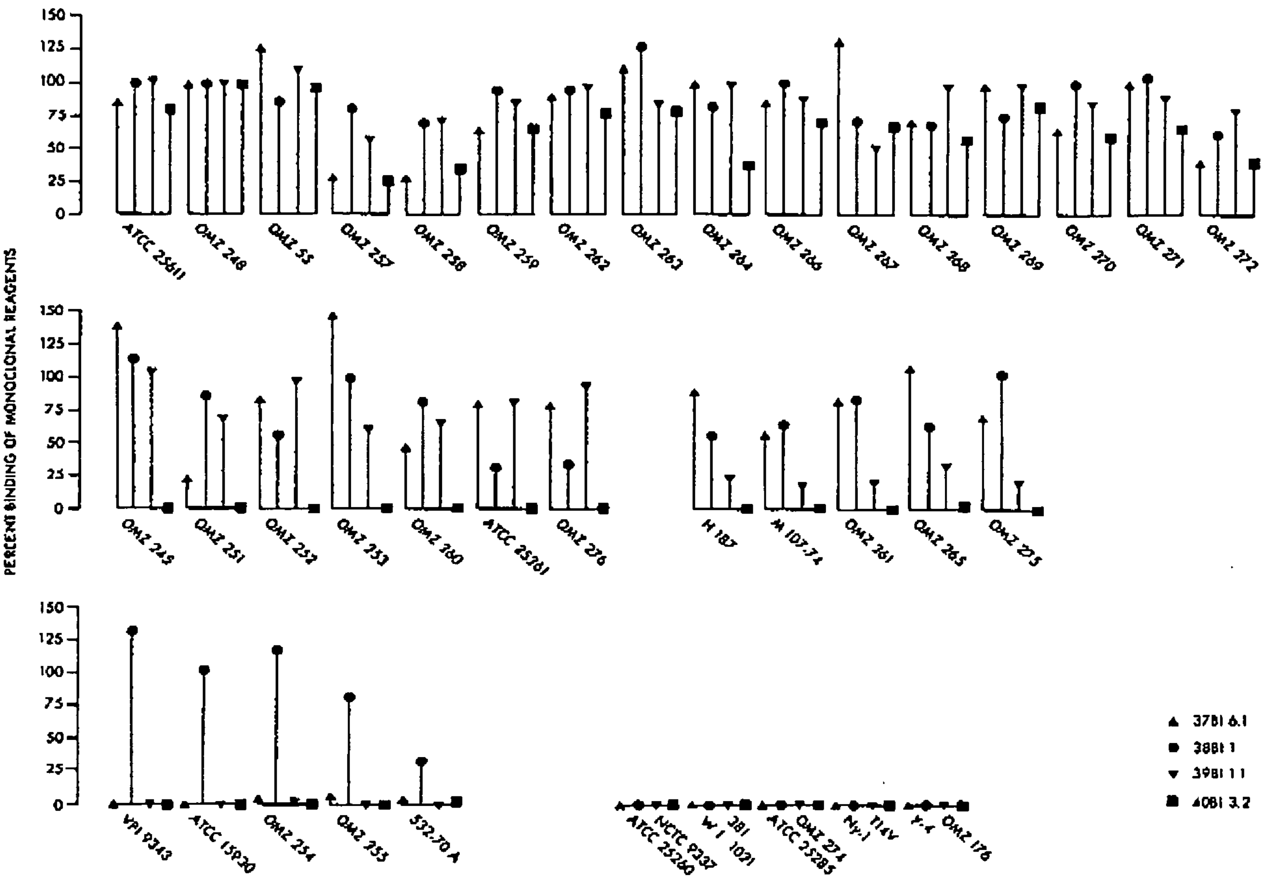

FIG. 2. Binding of the monoclonal antibodies 37BI6.1 (▲), 38BI1 (●), 39BI1.1 (▼), and 40BI3.2 (■) to 38 strains of various *Bacteroides* species and six strains from other genera (see the text). Antibody-binding was determined by ELISA with intact bacteria coated to microtest plates. The reactivity of each monoclonal antibody with the immunizing strain OMZ248 was arbitrarily defined as 100%.

trapping is shown in Fig. 3, which presents results from experiments in which still larger amounts of bacteria were used. Obviously, absorption of the monoclonal reagent followed a dose-dependent pattern unique for these three strains.

Table 3 provides a summary of the results presented thus far. Apparently the four monoclonal antibodies could react only with black-pigmenting strains that are either *B. intermedius* or belonged to the group of strains formerly classified as *B. melaninogenicus* subsp. *melaninogenicus*. They separated these strains into five distinct reactivity groups, three of which contained only *B. intermedius* strains of both oral and nonoral origins.

Metabolic properties of black-pigmenting strains used in this study. This obvious antigenic heterogeneity of the analyzed *B. intermedius* strains appears to contrast with the previously noted phenotypic homogeneity of the species. Since most of the *B. intermedius* strains used in this study were new and phenotypically not yet carefully characterized isolates, we decided to analyze them as well as the other black-pigmenting strains used for a number of characteristic metabolic properties. Results are summarized in Tables 4 and 5. All strains classified in the serological reactivity groups I, II, and III showed remarkably similar metabolic profiles (Table 4). By fermenting glucose but not lactose (two exceptions), hydrolizing gelatin (one exception) but not esculin, producing indole, acetic acid, isobutyric acid, and isovaleric acid and, at the most, only trace amounts of butyric acid, they exhibited the characteristic features of *B. intermedius*. The fermentation of lactose by two of the strains is exceptional. Nevertheless, we consider them as *B. intermedius* because of their other metabolical features. Furthermore, one of the two strains (H187) has been shown to belong to the *B. intermedius* DNA homology group B (26). Among the reactivity groups IV and V, four strains fermented glucose and lactose, hydrolyzed esculin, and were unable to produce indole. Two of these strains, ATCC15930 and VPI9343, have been very recently assigned to the two new *Bacteroides* species *B. loescheii*

TABLE 2. Absorption analysis of the expression of the antigens recognized by four monoclonal antibodies on black-pigmented *Bacteroides* strains

Strain	Monoclonal reagents[a]			
	37BI6.1	38BI1	39BI1.1	40BI3.2
OMZ248	72.7[b]	65.9	89.6	92.5
OMZ257	80.4	85.2	92.5	89.3
OMZ258	79.9	88.3	91.7	85.8
OMZ267	73.9	85.2	64.3	92.8
OMZ272	76.3	83.3	85.8	91.2
ATCC25261	100.0	100.0	82.5	0
OMZ251	76.7	67.0	95.8	4.4
OMZ260	75.6	64.2	70.6	0
OMZ276	68.9	44.0	88.5	0
M107-74	64.1	79.0	0	1.9
H187	58.3	66.4	3.0	0
OMZ261	67.4	46.0	2.4	1.3
OMZ265	78.1	75.4	8.1	0
OMZ275	79.2	74.6	0	0
VPI9343	8.3	100.0	0	0
ATCC15930	62.8	99.7	7.5	0
532-70A	0	88.5	0	1.7
OMZ254	35.0	96.3	0	3.5
OMZ255	66.2	92.3	1.7	0
NCTC9337	0	2.1	0	0
ATCC25260	0	6.7	7.2	0

[a] Hybridoma supernatants were absorbed at concentrations sufficient to yield 70 to 90% maximum binding in ELISA with strain OMZ248 bacteria as the antigen source.

[b] Absorptions were performed in triplicate with at least three concentrations of bacteria. However, for ease of comparison, data from only one concentration (1×) are shown. The ELISA data are expressed as percent reduction of binding of the monoclonal reagent to the target bacteria (strain OMZ248) after absorption with a given bacterial test strain.

and *B. melaninogenicus*, respectively (8), whereas the other two strains, OMZ254 and OMZ255, presently cannot definitely be classified because of the lack of DNA homology data. The five asaccharolytic strains included in this study were all well described reference strains. They did not express the antigens recognized by the monoclonal antibodies. Three of them are phenotypically characterized as *B. gingivalis* by their production of phenylacetic acid. The other two strains had the metabolic profile of *B. asaccharolyticus*, although with the unexpected feature of weak but significant glucose fermentation by strain ATCC25260.

DISCUSSION

In this paper we describe the production of four hybrid cell lines that produce monoclonal antibodies directed against antigens of *B. intermedius* OMZ248 and report the distribution of the recognized antigens on various strains of the genus *Bacteroides*. The few strains from other genera, namely *F. nucleatum*, *A. actinomycetemcomitans*, *A. viscosus*, and *S. mutans*, which were analyzed for the expression of these antigens, proved to be negative.

Analysis of the distribution of these antigens was performed with two different ELISA techniques. With the first technique, intact bacteria were coated onto the bottom of ELISA microtest plates and antigen expression was then assessed by the determination of the amount of monoclonal antibodies capable of binding to their target antigens. This method yields only semiquantitative results since the ability of the bacteria to coat the ELISA microtest plates varied from strain to strain. A characteristic feature of this approach is that antigen-antibody binding may occur under conditions of vast antibody excess, probably enabling the detection of antigens with only low affinity to the monoclonal antibodies. In a second set of experiments many strains were retested by quantitative absorption for expression of the antigens recognized by the four monoclonal antibodies. In contrast to the first technique, antigen-antibody binding occurs here under excess of antigen.

The two assays yielded different strain distributions for each of the four recognized antigens. It may be concluded, therefore, that the four monoclonal antibodies bind to four different molecules. The antigen detected by antibody 38BI1 was expressed by all saccharolytic black-pigmenting *Bacteroides* strains tested so far, regardless of whether they were isolates from oral or nonoral sites. Among the positive strains were the type strain (ATCC25611) of *B. interme-*

oclonal antibodies

40BI3.2
92.5
89.3
85.8
92.8
91.2
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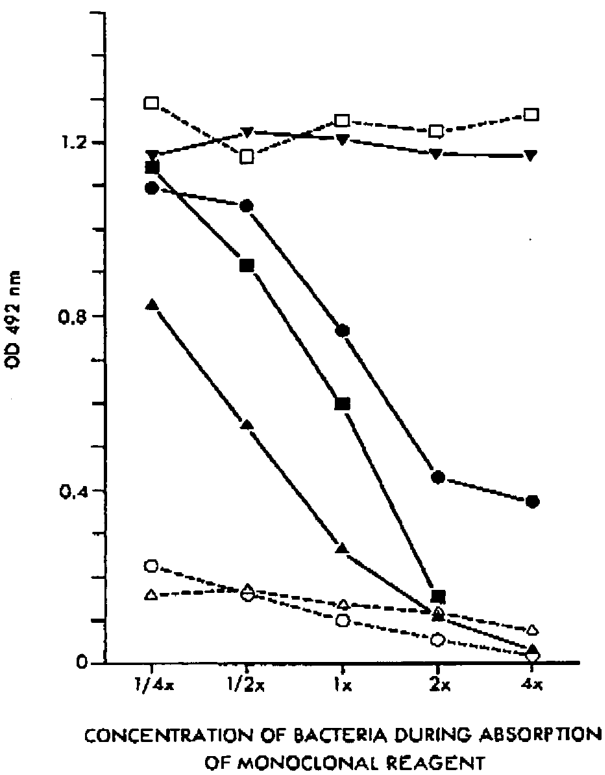

FIG. 3. Absorption analysis of reactivity of monoclonal antibody 37BI6.1 with bacteria from various strains. After absorption with M107-74 (○), 532-70A (□), OMZ265 (△), OMZ254 (●), OMZ255 (▲), ATCC15930 (■), or VPI9343 (▼), the residual binding activity of the monoclonal reagent was tested by ELISA on bacteria of strain OMZ248. The concentrations of bacteria used for absorption are defined in the text. Unabsorbed monoclonal reagent yielded in ELISA an optical density at 492 nm (OD 492 nm) of 1.175 ± 0.057 (standard deviation).

dius (9), the type strain (532-70A) of *B. corporis* (9), the type strain (ATCC15930) of *B. loescheii* (8), and a strain (VPI9343) belonging to the second DNA homology group of *B. melaninogenicus* (8). In contrast, the antigen could not be detected on any of five tested strains of the asaccharolytic black-pigmenting species *B. asaccharolyticus* and *B. gingivalis*, nor on the intestinal organism *B. fragilis*. It will certainly be of interest to analyze still more *Bacteroides* species (e.g., *B. denticola*, *B. oralis*, *B. levii*, and *B. macacae*) to determine how widespread this common antigen is. The detection of such an antigen with monoclonal antibodies is not surprising and confirms earlier observations of cross-reactivities among antisera raised against strains of *B. intermedius*, *B. corporis*, and *B. melaninogenicus* (11, 17, 20).

As for antibody 38BI1, monoclonal antibody 37BI6.1 recognized an antigen present on at least two different *Bacteroides* species. However, only *B. intermedius* strains expressed readily detectable amounts of the antigen. The frequency of expression among the strains of this species was 100%. Only very low amounts of the antigen were found on three strains, one of which was *B. loescheii*, whereas the two others have not been classified beyond belonging to the former *B. melaninogenicus* subsp. *melaninogenicus* group. The antigen levels on these three strains were too low to be detected in direct antibody binding tests, but absorption analysis of the monoclonal reagent with large amounts of intact bacteria yielded unambiguous, positive results.

The antigens defined by 39BI1.1 and 40BI3.2 are apparently restricted to *B. intermedius*. To our surprise, monoclonal antibody 39BI1.1 revealed two distinct types of dose-response curves with *B. intermedius* strains. Whereas for the majority of the strains, normal antibody binding was observed, a group of five strains could only be labeled in direct ELISA antibody-binding test at very high concentrations of antibody. Absorption tests with antibody 39BI1.1 and these strains, however, gave consistently negative results. At present, we do not have a satisfactory explanation for the seemingly contradictory results obtained with the two assay systems. The altered dose-response curves observed with five strains could be interpreted as reflecting expression of an antigen with a slightly altered antigenic binding site. We therefore operatively have designated this molecule as a cross-reactive antigen. Certainly, this hypothesis will have to be tested by additional experiments, which will require extensive purification of the target antigen of 39BI1.1.

Monoclonal antibody 40BI3.2 bound only to 57% of the *B. intermedius* strains tested, thereby separating the strains into two distinct groups. Taken together, the results on the relative expression of these antigens on various black-pigmenting *Bacteroides* strains suggest a separation of *B. intermedius* into two main reactivity groups (either 40BI3.2 positive or negative), of which one may be further subdivided on the basis of the different capacity of the strains to bind 39BI1.1 antibodies. In agreement with recently published genetic and immunological data (9, 20), nonoral isolates of *B. intermedius* did not reveal a specific antigenic profile, but rather proved to be phenotypically indistinguishable from oral strains in all parameters tested.

During the preparation of this manuscript, Johnson and Holdeman (9) proposed to divide th former *B. melaninogenicus* subsp. *intermedius* group into two new species, namely, *B. intermedius* and *B. corporis*. Although their data in support of such a division arose primarily from studies of DNA homologies, both metabol-

TABLE 3. Formation of reactivity groups based on the bacterial capacity to bind the monoclonal antibodies

Reactivity group	Strains	Expression of antigens recognized by monoclonal antibodies:			
		37B16.1	38B11	39B11.1	40B13.2
I	ATCC25611, OMZ55, 248, 257–259, 262–264, and 266–272	+	+	+	+
II	ATCC25261, OMZ245, 251–253, 260, and 276	+	+	+	−
III	H187, M107-74, OMZ261, 265, and 275	+	+	w[a]	−
IV	ATCC15930, OMZ254 and 255	+[b]	+	−	−
V	532-70A, VPI9343	−	+	−	−
VI	ATCC25260, NCTC9337, 381, 1021, W1, ATCC25285, Y-4, OMZ274, OMZ176, Ny1, T14V	−	−	−	−

[a] w, Expression of a weakly crossreacting antigen.
[b] Quantitatively very weak, but significant expression.

TABLE 4. Metabolic properties of *B. intermedius* strains used in this study

Metabolic properties[a]	Reaction					
	ATCC25611, OMZ248, 257–259, 262–264, 266, 267, and 269–272	OMZ 268	ATCC25261 OMZ252, 253, 260, and 276	OMZ251	OMZ261, 265, and 275	H187
Fermentation						
Glucose	+	+	+	+	+	+
Lactose	−	+	−	−	−	+
Hydrolysis						
Esculin	−	−	−	−	−	−
Gelatine	+	+	+	−	+	+
Starch	+	+	+	+	+	+
Proteolysis of milk	+	+	+	+	+	+
Production of:						
Indole	+	+	+	+	+	+
Lipase	+	+	+	+	+	+
Products from FUM						
Acetic acid	+	+	+	+	+	+
Isobutyric acid	+	+	+	+	+	+
Butyric acid[b]	−	−	−	−	−	−
Isovaleric acid	+	+	+	+	+	+
Valeric acid[c]	−	−	−	−	−	−
Phenylacetic acid	−	−	−	−	−	−
Reactivity group with monoclonal antibodies	I		II		III	

[a] All strains were gram negative, black pigmented on blood agar, unable to ferment mannitol or trehalose, and unable to produce catalase or lecithinase.
[b] Traces found with 69% of BI strains tested.
[c] Traces found with 10% of BI strains tested (OMZ260, 262, and 263).

TABLE 5. Metabolic properties of black-pigmenting *Bacteroides* strains used in this study (excluding *B. intermedius*)

Metabolic properties[a]	Reaction						
	B. loescheii ATCC15930	Unidentified OMZ254	Unidentified OMZ 255	*B. melaninogenicus* VPI9343	*B. corporis* 532-70A	*B. asaccharolyticus* NTCC 9337 and ATCC 25260	*B. gingivalis* W1, 1021, and 381
Fermentation							
Glucose	+	+	+	+	+	±[b]	−
Lactose	+	+	+	+	−	−	−
Hydrolysis							
Esculin	+	+	+	+	−	−	−
Gelatine	−	−	−	−	−	+	+
Starch	−	+	+	+	−	−	−
Proteolysis of milk	−	+	−	−	−	+	+
Production of:							
Indole	−	−	−	−	−	+	+
Lipase	+	+	−	−	−	−	−
Products from FUM							
Acetic acid	+	+	+	+	+	+	+
Isobutyric acid	−	−[b]	+	+	+	+	+
Butyric acid	−[b]	−[b]	−	−[b]	−	+	+
Isovaleric acid	−[b]	−[b]	+	+	+	+	+
Valeric acid	−	−	−	−	−	−	−
Phenylacetic acid	−	−	−	−	−	−	+
Reactivity groups with monoclonal antibodies		IV		V		VI	

[a] All strains were gram negative, black or brown pigmented on blood agar, unable to ferment mannitol or trehalose, and unable to produce catalase or lecithinase.

[b] Trace amounts were produced.

ic and antigenic differences between strains of the new species have been known for several years (11) and were confirmed again in this study. More interestingly, however, was that the strains united in the new species *B. intermedius* separated into two genetically distinct but phenotypically indistinguishable DNA homology groups (9). Remarkably, the results presented in this paper divide *B. intermedius* strains into two main groups (either 40BI3.2 positive or negative) as well. It is obvious to ask now whether the antigen recognized by monoclonal antibody 40BI3.2 is a phenotypic marker of strains belonging to one of the two DNA homology groups. If so, this would allow the rapid classification of new isolates (e.g., by immunofluorescence) without the necessity of laborious DNA homology studies. We cannot yet answer the question because for only three of the *B. intermedius* strains analyzed in our study is the DNA homology group known. Nev rtheless, the classifications of these three strains obtained either by DNA homology or, alternativ ly, by 40BI3.2 monoclonal antibody typing are in remarkable agreement; strain ATCC25611 has been reported to belong to the *B. intermedius* homology group I (9, 26) and is shown in this paper to fall into reactivity group I (expressing all the recognized antigens), whereas strains ATCC25261 and H187 are known to be part of DNA homology group II (9, 26), and both failed to express the 40BI3.2 defined antigen. In view of these results, we tentatively describe reactivity groups I, II, and III as three new serogroups of *B. intermedius*. Certainly, further studies will be necessary to determine whether this promising correlation of genetic and phenotypic parameters is true for all *B. intermedius* strains. In the affirmative case, the use of monoclonal antibodies 40BI3.2 and 39BI1.1 as markers of new serogroups will be justified and probably very helpful.

In conclusion, we have produced a set of monoclonal antibodies that define four independent *B. intermedius* antigens partially shared by other black-pigmenting saccharolytic *Bacteroides* species. The expression of the antigens by the strains is not random but specific and may

serve as an indicator of their antigenic relationship. None of the antigens has been biochemically characterized. Nevertheless, our observations that all four antigens can be readily detected by direct labeling of intact bacteria with the monoclonal antibodies or by absorption of the antibodies with freshly collected bacteria strongly suggest that the antigens are located in the outer membrane or in the layer covering this structure. Although not demonstrated here, the ease with which the antigens can be detected in immunofluorescence tests could make these monoclonal antibodies interesting tools for the identification of black-pigmenting saccharolytic *Bacteroides* isolates, in particular the species *B. intermedius*.

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